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PART (1) THE ROLE OF GLUCURONIDE METABOLISM IN THE GROWTH OF ANIMAL TISSUES

(2) A GLUCURONIDE-DECOMPOSING ENZYME IN THE RUMEN OF THE SHEEP

PART (1)

THE ROLE OF GLUCURONIDE METABOLISM IN THE GROWTH OF ANIMAL TISSUES

C O N T E N T S

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The enzyme, β-glucuronidase, present in mammalian tissues, catalyses the breakdown of β-d-glucuronides *in vitro* to glucuronic acid and the corresponding "aglucone". Although evidence for its presence was obtained by Roehmann in 1908 and by Sera in 1914, it was not till fairly recently that this enzyme was studied in any detail.

Roehmann observed that a chloroform water extract of dog liver was capable of destroying menthol-β-d-glucuronide. He followed the disappearance of this substance by measuring changes in the rotation of polarized light by the solution. Using the same technique, Sera extended these observations to other glucuronides, using extracts from liver and other organs of animals besides the dog. As both workers merely measured changes in the optical activity of the solutions undergoing the transformation, they were unable to throw any light on the nature of the reaction - although tentatively they suggested a hydrolysis of the glucuronide to glucuronic acid and the corresponding "aglucone".

About twenty years later - in 1934 - the Japanese worker, Masamune, demonstrated the presence of an enzyme in mammalian tissues capable of hydrolysing bio-synthetic β-d-glucuronides. He prepared an extract of ox-kidney and showed that it was able to hydrolyse menthol-β-d-glucuronide to menthol and glucuronic acid.

He measured the free menthol colorimetrically and the glucuronic acid by its reducing power. His evidence for glucuronic acid being one of the reaction products was based on the fact that the de-proteinized filtrate from his reaction mixture showed the naphtho resorcinol reaction of Neuberg and Sane Yoshi for glucuronic acid and the Wheeler and Tollens phloroglucinol test for pentoses.

The enzyme extract was shown to have the following properties:-

1). It hydrolysed several bio-synthetic glucuronides. These same glucuronides were also broken down, although slowly, by emulsin - an enzyme preparation specific for the β-oside linkage.

2). The enzyme extract had no effect on β-glucosides except β-phenyl glucoside.

3). It had no effect on synthetic α-menthol glucuronide.

4). The amount of glucuronic acid formed from menthol glucuronide during the reaction - as measured by its reducing power - was equivalent to the amount of free menthol produced - within experimental errors.

5). The pH optimum of this enzyme lay between 5.3 - 5.6 for the various substrates tested. That for emulsin was about pH 4. Furthermore, the degree of hydrolysis of the glucuronides by emulsin was much lower than that of the β-glucosides in a

given time.

From these observations, Masamune concluded that the enzyme he was studying was one specific for the hydrolysis of β-d-glucuronides and that glucuronic acid and the corresponding "aglucone" were the two products of this reaction. He termed this enzyme β-glucuronosidase.

Oshima (1934, 1936), continuing the work of Masamune developed a slightly better method of extracting and purifying the enzyme. This procedure involved adsorption of the enzyme on kaolin at a suitable pH and subsequent elution with phosphate buffer. He examined the distribution of the enzyme in the various tissues of the ox and the dog, and found that it was fairly extensively distributed. The enzyme activity was found to be particularly high in spleen and liver. His kinetic studies on the enzyme confirmed Masamune's finding that the pH optimum was about 5.2 for menthol glucuronide. The values obtained by him for the velocity constant suggested that the reaction was monomolecular. Finally, he observed that certain hydroxy dicarboxylic acids such as malic, tartaric and citric acids had an inhibitory effect on the enzyme activity, while certain other acids like lactic, acetic, propionic and butyric appeared to enhance the activity.

Considerable attention has been paid to the preparation and purification of fairly active enzyme fractions by subsequent workers. Masamune minced the fat freed ox-kidney, mixed it with twice its weight of 0.85%

saline, a suitable quantity of toluene and incubated the mixture at 38°C for 3 days. The resulting autolysate was centrifuged, the supernatant cleared with diatomaceous earth and centrifuged again. The clear liquid was poured into 3 volumes of alcohol and the precipitate formed centrifuged as quickly as possible. The precipitate was pressed between filter papers and subsequently extracted with a suitable quantity of water. The aqueous enzyme extract was treated with N. H_2SO_4, the protein precipitated centrifuged off and the supernatant treated as before with alcohol. The final aqueous extract was quite clear.

Oshima followed the method of Masamune in the earlier stages. He adjusted the aqueous extract to pH 3.6 and adsorbed the enzyme on kaolin and then eluted it with M/15 Na_2. H PO_4. The solution was then dialysed. The dialysed solution had 60% of the original activity and 30% of N content, thus resulting in a two-fold concentration and purification of the enzyme.

Fishman (1939) achieved a 140-fold purification of the enzyme by precipitating it from a crude aqueous extract of the minced tissue, with acetone. This precipitate was redissolved in water and fractionated with ammonium sulphate at a suitable pH. Preliminary treatment involved clearing of the turbid aqueous extract with diatomaceous earth.

Graham (1946) observed that acetone precipitation of an aqueous extract caused a loss in enzyme activity while an aqueous extraction of acetone dried minced

ox spleen decreased this loss in activity. By incubating this aqueous extract at pH 5.0 and 25-30°C for 6 hours, followed by centrifuging down precipitated inactive protein and fractionation of the supernatant with ammonium sulphate at pH 4.3, 5.0 and 7.3, he achieved a 300-fold purification of the enzyme. His attempts to crystallize the enzyme however proved unsuccessful.

The earlier workers (Masamune 1934, Oshima 1934, 1936, Fishman 1939, 1940, 1944, 1947, Graham 1946 and Mills 1946) estimated the enzyme activity by measuring the reducing material formed on incubation of the enzyme preparation with a suitable glucuronide. The use of such methods rested on the assumption that the glucuronides were broken down to glucuronic acid and the corresponding "aglucone". The evidence for this was indirect and not conclusive. Levvy (1948) isolated glucuronic acid - liberated from menthol glucuronide by enzyme hydrolysis - and characterised it as the dibenziminazole derivative of saccharic acid. From enzyme initially hydrolysed menthol glucuronide solutions, glucuronic acid was separated from the unchanged glucuronide and estimated by the naphthoresorcinol colour reaction (Hanson et al. 1944). These values agreed with those obtained by the ferricyanide reduction method (Levvy 1946) suggesting that the earlier methods based on the estimation of reducing material were reliable for assays of enzyme activity. Masamune's assumption that the breakdown of the glucuronide at the oside link gave glucuronic acid was confirmed by Levvy's results.

With crude enzyme preparations however, a large amount of reducing material - which was not glucuronic acid - was produced when the enzyme was incubated in the absence of the substrate. (Graham 1946, Levvy 1948). This resulted in large enzyme blanks which were unsatisfactory and at times made estimations unreliable. This led Fishman and his co-workers (1946) to develop a method where the "aglucone" was measured colorimetrically. They used phenolphthalein glucuronide as substrate. The freed phenolphthalein was measured colorimetrically after the reaction mixture had been brought to pH 10.45 with glycine buffer. This method appears to avoid satisfactorily the difficulties associated with measurements of reducing power.

About the same time, Kerr et al. (1948) developed an assay procedure using phenyl-glucuronide as substrate. The liberated phenol was measured by a modified King and Armstrong method (1934) using the Folin & Ciocalteau reagent (1927). While this method is quite useful with purified enzyme preparations (Fishman and Anlyan 1947) it is not very satisfactory when crude tissue homogenates or enzyme preparations are used. The non-specificity of the phenol colour reagent results in high enzyme blanks with crude enzyme preparations.

A hydrolytic enzyme should catalyse a reaction in either direction. It was therefore quite natural for the earlier workers in this field (Oshima, 1936, Fishman, 1940) to postulate that the function of β-glucuronidase in vivo was the synthesis of glucuronides.

This view appeared to be supported by the fact that man and other animals were known to excrete glucuronides in the urine. Fishman (1940) carried out experiments in vivo with a view to finding out the function of β-glucuronidase in the animal body. Feeding of glucuronidogenic substances to dogs has been shown to result in increased glucuronide excretion in urine (Quick 1926). Pryde and Williams (1934) have shown that mice excrete conjugated glucuronic acids in the urine. Consequently Fishman administered borneol to dogs and menthol to mice and measured the glucuronidase activity in liver, kidney and other organs. Both these compounds are glucuronidogenic. He observed rises in the enzyme activity in liver, kidney and spleen, but not in pancreas, ovary, testes, uterus or vagina. Fishman concluded that the increased glucuronidase activity was in response to a greater demand for glucuronide synthesis. In later experiments (1944, 1947) where he administered estrogenic substances to mice he observed a rise in enzyme activity in uterus but not in liver, even though large doses were administered. Oestriol and stilboestrol are known to form glucuronides. To explain these results Fishman had to postulate two types of β-glucuronidase - one showing a response to oestrogens and the other responding to other glucuronidogenic substances in general.

In this laboratory, Levvy and his co-workers (Levvy et al. 1948, Kerr et al. 1949) have re-investigated the problem. They found similar rises in liver glucuronidase activity in the mouse following

administration of menthol. But closely associated with the rise in enzyme activity was observed a state of rapid cell proliferation in the tissues examined. They also found that this effect was not solely confined to the administration of glucuronidogenic compounds - which were at the same time toxic - but also to other compounds such as chloroform, carbon tetrachloride, and phosphorus, which were not glucuronidogenic but toxic. They therefore concluded that a rise in glucuronidase activity in a tissue was associated with a rapid state of cell proliferation in the tissue and not a response to increased demand for glucuronide synthesis. They extended these observations also to kidney and uterus in the mouse. Their hypothesis obtained further support when high glucuronidase activities were noted in infant mouse liver, kidney, lung and uterus, in regenerating liver after partial hepatectomy (Levvy et al. 1948, Kerr et al. 1949) and in cancer tissues (Fishman and Anlyan 1947). In all these cases the tissues are in a state of active cell proliferation. Administration of colchicine, which is a mitotic poison, has been shown by Kerr et al. (1950) to lower the glucuronidase activity in the liver of young mice and to prevent the rise in liver glucuronidase activity after partial hepatectomy. All these results and Fishman's could be easily explained on Levvy's hypothesis. Furthermore, there was no need to postulate two physiologically different enzymes as Fishman had suggested. That such a hypothesis is needless is further stressed by the observation of Kerr et al. (1950) that in ovariectomized mice the administration of carbon

tetrachloride or chloroform causes a rise in the glucuronidase activity of the uterus while oestrone causes a rise in that of liver. These rises were associated with parallel changes in the mitotic activity of the tissues.

The weight of evidence available would therefore appear to make Fishman's postulated role for β-glucuronidase *in vivo* untenable. The ability of tissue slices to form glucuronides when incubated with suitable glucuronidogenic substances has been demonstrated by several workers (Lipschitz and Bueding 1939, Bueding and Ladewig 1939, Kensler *et al.* 1941, De Meio and Arnolt 1944, Odette Crepy 1946). Although the enzyme system responsible for glucuronide synthesis has not been characterized several important observations have been made.

Lipschitz and Bueding incubated tissue slices from guinea pig - in the presence of borneol or menthol and estimated the glucuronide formed, after separating it from other interfering substances, by the Tollens colour reaction. The method was cumbersome but nevertheless yielded valuable results in their hands. They concluded from their observations that the formation of glucuronides was confined mainly to liver, that oxidation was an essential process for glucuronide formation, that a phosphorylating mechanism was involved, and that certain 3-carbon compounds stimulated the production of conjugated glucuronides, whilst addition of glucuronic acid, glucosides or maltosides had no effect.

De Meio and Arnolt (1944) have studied

the conjugation of phenol by rat and cat tissue slices. They estimated the amount of phenol conjugated by a modified Theis and Benedict method. The method has been criticised by Levvy and Storey (1949) as giving no indication of glucuronide formation and hence their results are open to doubt.

Though other workers have shown that liver slices are capable of conjugating glucuronides in the presence of suitable aglucones the mechanism of glucuronide formation still remains unsolved.

Florkin et al (1942) attempted an enzyme synthesis of borneol glucuronide by incubating borneol, glucuronic acid and ox spleen β-glucuronidase preparation together for long periods. They estimated the glucuronide formed by a modified Tollens method. Several days incubation produced such small conjugation that the authors themselves doubted whether this could possibly be the mechanism operating in vivo.

The present work was started with a view to finding possible inhibitors for β-glucuronidase and making use of the inhibitors in an attempt to elucidate the possible physiological role of this enzyme in the animal body. A powerful inhibitor was found in D-glucosaccharic acid. Although it served a useful purpose in the in vitro studies it proved ineffective in the in vivo experiments. The nature of the compound would suggest that it was easily metabolised in the living animal.

The glucuronide synthesizing system

in the mouse has been studied using the relatively simple but reliable method of Levvy and Storey (1949). Both normal and treated animals have been used. The results obtained suggest that changes in the glucuronidase activity in the liver bear no relationship to changes in its glucuronide synthesising activity. The two enzyme systems appear to be quite distinct from one another.

SECTION (i)

THE INHIBITION OF β-GLUCURONIDASE BY SACCHARIC ACID AND OTHER SUBSTANCES

INTRODUCTION

The physiological functions of several enzymes have been elucidated by studying the effect of inhibitors on their action both *in vivo* and *in vitro*. Although the most valuable information yielded by this technique has been obtained with enzyme systems other than hydrolytic ones, it was felt that if a suitable specific inhibitor could be obtained for β-glucuronidase, it might prove useful in elucidating its physiological function. Fishman (1940) had suggested, though he had no direct evidence to support his view, that β-glucuronidase was responsible for the formation of glucuronides *in vivo*. The evidence for this hypothesis was the increased glucuronidase activity observed in the liver and uterus of dogs and mice which had received glucuronidogenic substances (1940, 1944, 1947). Levvy *et al*. (1948) have, however, shown that this view was not necessarily valid as Fishman's results and theirs could be interpreted on the hypothesis that increased glucuronidase activity reflects increased tissue proliferation.

It was felt that the use of a specific inhibitor might help to clear up these points. Consequently several known inhibitors of enzymes and other substances were tested for their inhibitory power, resulting in the discovery of D-glucosaccharic acid - the most effective inhibitor so far obtained. A few others, less powerful in their action, were

also discovered in the course of this work.

The effect of saccharic acid *in vivo* has been examined in the mouse. The results obtained have so far been negative.

EXPERIMENTAL

Preparation of phenyl-β-d-glucuronide

Phenyl-β-d-glucuronide was prepared biosynthetically by feeding rabbits a 5% (w/v) aqueous solution of phenol. The urine was collected over a period of 18 hours and the excreted phenylglucuronide isolated from the urine through its basic lead salt. (Porteus & Williams 1949). The phenylglucuronide finally recrystallised from a mixture of benzene and ethyl alcohol was a white solid. After drying over P_2O_5 *in vacuo* at 80°C for 10 hours, the compound had a m.p. of 162°C (Corr.).

Preparation of enzyme for assay

The animal was killed by a blow on the head and the organ required was dissected out rapidly. It was freed of connective tissue as much as possible. With kidneys, the outer skin was removed before further treatment. The weighed organ was homogenized in a chilled glass homogenizer (Umbreit, Burris & Stauffer, 1945, p.92) using a suitable quantity of water. The homogenate was adjusted to pH 5.2 by adding 0.3 M citrate buffer, bringing the final concentration of buffer to 0.03 M. The homogenate was

incubated at 37° C for ½ hr. and insoluble material centrifuged down. The supernatant was fractionated with ammonium sulphate - the fraction precipitated between 31.5 - 50% saturation being the active glucuronidase fraction. The precipitate was dissolved in a suitable quantity of distilled water - about 4 mls/gm liver tissue.

In certain experiments, the crude homogenate was used for assay, while in others the cell-free supernatant was used without any ammonium sulphate fractionation. In a few experiments, the two glucuronidase fractions described by Mills (1948) were prepared. Fraction (A) was precipitated between 31.5 - 38.5% saturation with ammonium sulphate while fraction (B) was precipitated between 38.5 - 50% saturation.

Preparation of inhibitor solutions

Aqueous solutions of the substances were prepared whenever possible. The pH of the solution was adjusted to 5.2 using a glass electrode. With substances insoluble or sparingly soluble in water, an alcohol-water mixture or, as in one instance, ethylene glycol was used as solvents. With such solutions, suitable controls were carried out for any possible effect of the solvent on the hydrolysis.

Determination of β-glucuronidase activity

The hydrolysis of phenylglucuronide by mouse liver or kidney glucuronidase preparations was measured

by the procedure of Kerr, Graham & Levvy (1948). In testing substances for their inhibitory action on the enzyme, incubation mixtures were made up as follows:-

0.4 ml	Enzyme preparation
0.2 ml	0.1 M Citrate buffer pH 5.2
0.1 ml	0.12 M Phenylglucuronide
0.1 ml	Inhibitor solution

In controls, water was substituted for the inhibitor solution. Substrate and inhibitor solutions were adjusted to pH 5.2 (glass electrode). The necessary incubation mixture for enzyme, substrate and inhibitor blanks were also set up. At the end of 1 hour's incubation, the enzyme action was stopped by the addition of Folin and Cio-calteau reagent, and the colour of the liberated phenol developed with sodium carbonate solution as described in the procedure of Kerr et al.

Mixed blanks for the effect of the inhibitor on either the substrate or enzyme blanks were also set up.

In experiments in which the two β-glucuronidase fractions (A) and (B) were examined separately hydrolysis with fraction (A) was carried out at pH 4.5 instead of at 5.2.

Mouse uterus has only one glucuronidase fraction -(Kerr et al. 1949). This corresponds to fraction (A) in liver and assays with uterine enzyme preparations were therefore done at pH 4.5.

Results are usually expressed as μg of phenol liberated per hour at 37° C.

RESULTS

A) The action of various substances on β-glucuronidase activity

Nearly fifty substances were examined for their effect on β-glucuronidase activity in vitro. Some of them were known inhibitors of enzyme systems whilst others bore a structural resemblance to a β-glucuronide. Those substances which caused an inhibition of enzyme activity are shown in table (1).

Table 1. Inhibition of mouse liver β-glucuronidase in vitro (0.015 M-phenylglucuronide)

Compound	Concentration (10^{-4}M)	Phenol liberated		Inhibition (%)
		In controls (μg.)	In presence of inhibitor (μg.)	
Saccharic acid	150	32.1	3.3	90
Mucic acid	75	30.2	23.6	22
D-Gluconic acid	150	37.4	28.8	23
D-Glucurone ✱	300	40.0	0	100
	150	35.3	12.1	66
	38	55.2	32.4	41
	10	55.2	45.2	18
	3.3	55.2	51.2	7
L-Malic acid ‡	150	23.7	14.9	37
DL-Malic acid	300	32.9	18.6	44
	150	30.2	23.5	22
Phlorrhizin ☒	3	16.3	13.6	17
	1.5	16.3	16.8	-3
Vanillin ☒	7.5	39.4	28.8	27

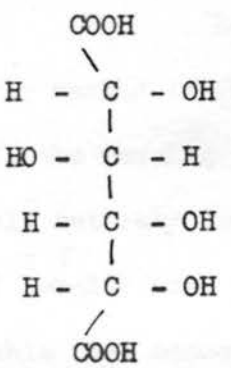
¥ Interferes in colour reaction for phenol. Results are corrected for interference.

‡ The naturally occurring isomer, commonly called laevorotatory malic acid.

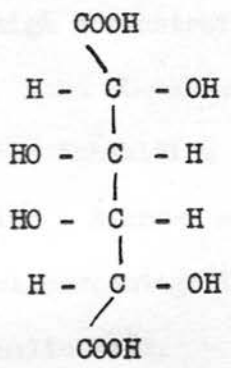
§ Gives colour with phenol reagent. Results are corrected for this colour.

Of these, the most powerful inhibitor - D-gluco saccharic acid (hereinafter referred to as saccharic acid) has been studied in some detail. Of the other compounds listed in table (1), mucic, gluconic and glucuronic acids were closely related to saccharic acid. Nevertheless they were much less efficient as inhibitors of β-glucuronidase.

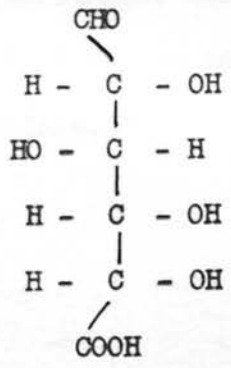
Structural formulae



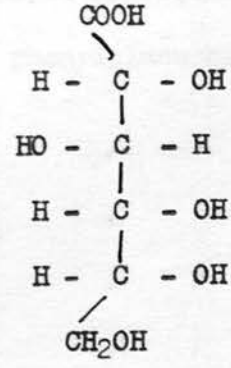
Saccharic acid



Mucic acid



Glucuronic acid



Gluconic acid

Glucuronic acid (D-glucurone) interfered in the determination of phenol liberated from phenylglucuronide. Glucuronic acid by itself does not give a colour with the Folin-Cio-calteau reagent, but in the presence of phenol or phenylglucuronide it increases by a small amount the colour given either by the free phenol or phenyl-glucuronide. The amount of increase in colour was apparently independant of the concentration of phenol. The figures in table (1) have been corrected for this interference and are considered reliable.

Phlorrhizin and vanillin gave colours directly with the Folin and Cio-calteau reagent. After correction for this, the results suggested that both these compounds inhibited β-glucuronidase very slightly.

L-Malic acid in high concentrations has a fairly marked inhibitory action. When DL-malic acid was tested, the results suggested that the inhibition produced was probably entirely due to L-malic acid. A known concentration of L-malic acid produced the same percentage inhibition as did double that concentration of DL-malic acid.

The accompanying tables list the remaining substances that have been examined and found to have no apparent effect on the hydrolysis of phenylglucuronide by β-glucuronidase.

Table (2)

List of substances which had no apparent effect on the hydrolysis of phenylglucuronide by β-glucuronidase in the concentrations shown.

Substance	Concentration	% Inhibition
β-Phenyl-D-glucoside	0.015 M	-4
α-Methyl-D-glucoside	0.015 M	-7
α-Methyl-D-mannoside	0.003 M	7
β-Methyl-D-glucoheptoside	0.003 M	0
β-Methyl-D-xyloside	0.003 M	1
α-Methyl-D-galactoside	0.003 M	4
Gum arabic	0.15%	7
Degraded egg plum gum	0.05%	4
Pyromucic acid	0.015 M	7
Sorbic acid	0.02 M	6
Oxalic acid	0.015 M	-3
Malonic acid	0.015 M	-4
Succinic acid	0.015 M	0
Glutaric acid	0.015 M	3
Maleic acid	0.015 M	-4
L-Tartaric acid	0.015 M	2
Valeric acid	0.015 M	-6
Ouabain	0.0015 M	-4
Digitonin	0.0015 M	0
Urethane	0.015 M	0
* Phenylurethane	0.015 M	-18

Table (2) - Contd.

Substance	Concentration	% Inhibition
✱ Nitroso-N-methylurethane	0.015 M	-7
Heparin	6.6 Toronto units/ml	-6
Sulphapyridine	0.00015 M	2
Inositol	0.015 M	4
‡ Piperonal	0.015 M	-10
‡ n-Hexyl alcohol	0.015 M	-6
NN-di-(2-chloroethyl)-aniline	0.02 M	0
2'-Methyl-4-dimethylaminostilbene	0.01 M	0
Sodium fluoride	0.015 M	-2
Sodium sulphate	0.03 M	-2
⁂ Di-isopropofluorophosphonate	0.002 M	-10
✱ Safrol	0.015 M	-2
Iodoacetic acid	0.005 M	0
Menthol	0.0006 M	0
Dinitrophenol	0.001 M	7

✱ Ethyl Alcohol and water

‡ Ethyl Alcohol

⁂ Ethylene glycol

Table(3)

The following compounds gave colours with the phenol reagent, but after correction had been made for this they were apparently without effect on β-glucuronidase.

Compound	Concentration	% Inhibition
Salicin	0.015 M	-4
Thiourea	0.0015 M	-4
Eserine	0.0001 M	9
Ascorbic acid	0.00075 M	0
‡ Oestrone	0.0005 M	5
Colchicine	0.0001 M	0

‡ Ethyl alcohol

The following compounds interfered too badly in the colour reaction to be tested with β-glucuronidase:-

Sodium azide

w-bromoacetophenone

Phenylarsenoxide

Ethylcyanoacetate

Ethane- 1:2-dithiol

Salol

With these compounds when corrections for the interference in colour were attempted, the resulting hydrolysis values showed marked activation.

B) Action of saccharic acid on β-glucuronidase

Saccharic acid being the most powerful of the inhibitors studied so far was examined in some detail. The effect of varying concentrations of saccharic acid on mouse liver glucuronidase activity was studied. The results of three different experiments are shown graphically in Fig. (1.). The concentrations are plotted on a logarithmic scale. The percentage inhibition rises very slowly from 10^{-6} to 10^{-5}M saccharate concentration and then steeply between 10^{-5} and 10^{-3}M and then slowly again beyond 10^{-3}M concentration. Almost complete inhibition is caused by 10^{-2}M saccharate while 50% inhibition was obtained with 2×10^{-4}M concentration of saccharate.

Fig. (1)

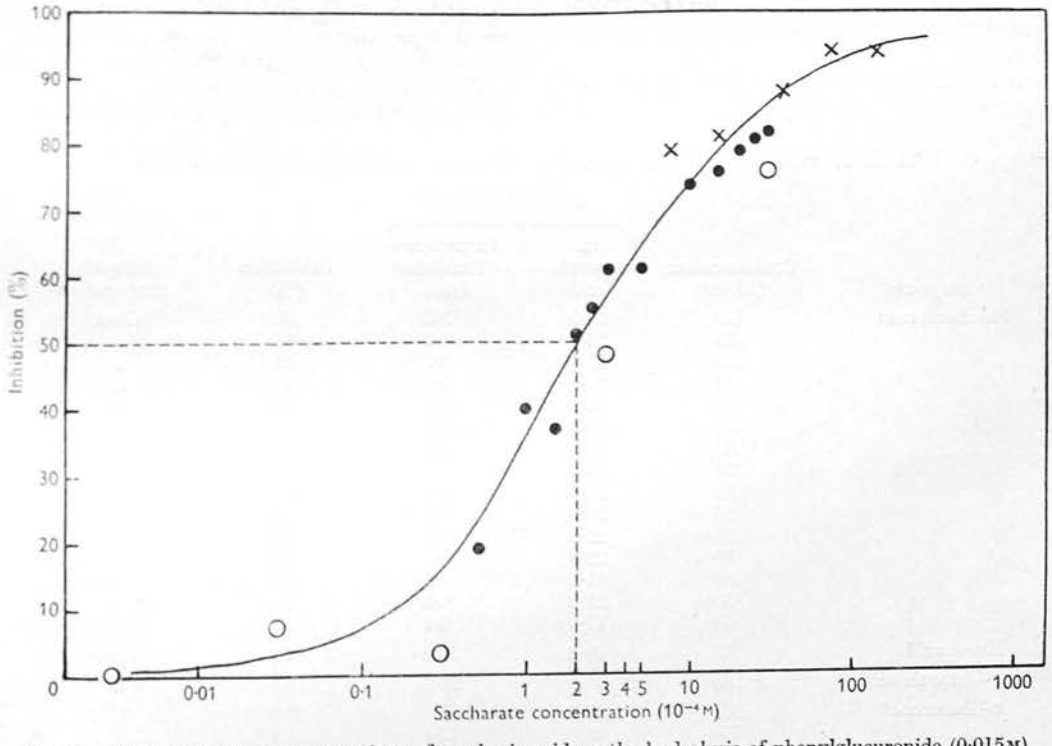


Fig. 1. Effect of varying concentrations of saccharic acid on the hydrolysis of phenylglucuronide (0·015M) by mouse-liver glucuronidase (results for three separate experiments shown by ○, ● and ×).

Table (4) shows the concentrations of a few inhibitors causing 20% inhibition of β-glucuronidase activity. It is quite clear that of the substances studied saccharic acid is by far the most powerful.

Table (4)

Concentrations of some compounds causing 20% inhibition of β-glucuronidase activity

Compound	Conc. ($\times 10^{-4}$M)
Saccharic acid	0.4
Gluconic acid	150
Mucic acid	75
L-malic acid	75
Glucuronic acid (glucurone)	10

C) Effect of saccharic acid on β-glucuronidase preparations from different organs of the mouse

The action of saccharic acid on β-glucuronidase preparations from different organs was studied. The organs examined were the liver, kidney, uterus and spleen - these being organs with high glucuronidase activities. Enzyme preparations in varying degress of purification have been examined. In some instances crude homogenate of the tissues have been used. In others, the crude homogenates have been centrifuged to give a cell-free aqueous extract. In still others, this cell-free supernatant has been fractionated

with ammonium sulphate either to give total glucuronidase (AB) or fractions A and B separately.

Table (5) lists the results obtained in these experiments. It would appear from the results that the inhibition by saccharic acid was independent of the source of the enzyme or the extent of its purification.

Table (5)

Effect of saccharic acid on β-glucuronidase preparations from different organs of the mouse.

Organ	Nature of enzyme preparation	Conctn. of saccharic acid $x(10^{-4}M)$	Hydrolysis (phenol liberated)		% Inhibition
			In controls (μg)	In presence of inhibitor (μg)	
Liver	Crude homogenate	50	22.5	0.5	98
	Am_2SO_4 pptd. fraction (AB)	50	21.2	3.2	85
	Fraction A	50	20.5	4.6	78
	Fraction B	50	22.8	3.4	85
Infant mouse liver	Cell-free aqueous homogenate	100	27.5	1.3	95
Kidney	Crude homogenate	50	17.0	5.7	67
	Am_2SO_4 pptd. fraction AB	50	21.2	6.5	70
Uterus	Cell-free aqueous homogenate	100	14.8	0.8	95
Spleen	Cell-free aqueous homogenate	100	20.5	1.0	95

D) Effect of saccharic acid on phenol colour development

Besides the usual controls set up during an estimation for any possible effect the inhibitor may have on the enzyme or substrate blanks, the effect of saccharic acid on phenol colour development was studied. Incubation tubes were set up containing buffer, saccharate and phenol solutions. In the controls, the saccharate solution was replaced by an equal volume of water. The final concentration of saccharate was 0.015 M. After 1 hour's incubation at 37° C, the colours were developed in the usual manner. Table (6) shows percentage recoveries obtained at varying phenol concentrations.

Table (6)

Percentage recoveries of phenol in the presence of 0.015 M saccharic acid

µg Phenol estimated		% Recovery
In Controls	In presence of inhibitor	
29.4	31.3	107
39.2	42.5	108
51.2	53.2	104
66.5	66.7	100
84.2	84.2	100

It would appear from the percentage recoveries obtained that

the small correction to be applied would tend to increase the values obtained for percentage inhibition very slightly. For most purposes this small correction could be ignored.

E) Effect of enzyme concentration on percentage inhibition caused by saccharic acid

The possibility of the potency of an enzyme preparation having an influence on the percentage inhibition caused by a known concentration of saccharic acid was investigated. Varying dilutions of the same enzyme preparation were made. The percentage inhibition by approx. 10^{-4}M saccharate on these enzyme preparations was then estimated by the usual procedure. Table (7) summarizes the results of a typical experiment.

Table (7)

The effect of saccharic acid ($\simeq 10^{-4}$M) on the hydrolysis caused by varying concentrations of β-glucuronidase preparations

Enzyme preparation	Hydrolysis		% inhibition
	In controls (μg)	In presence of saccharate (μg)	
(A)	40.5	17.6	56
(B)	32.5	12.3	62
(C)	19.5	8.0	59
(D)	10.2	4.0	61

Concentrations of enzyme solutions were approx.

(A) = $\frac{4}{3}$ (B) = 2 (C) = 4 (D)

The results suggest that within the limits of enzyme dilution studied, the variation in the percentage inhibition obtained with varying enzyme dilutions was not significant.

F) The reversibility of saccharate inhibition

From its similarity in structure to glucuronic acid, one would expect saccharic acid to act competitively in inhibiting glucuronidase. It might be expected to form a complex with the enzyme masking the active centres on the enzyme protein normally occupied by the substrate molecule. In the case of a competitive inhibitor this combination would very likely be a loose one. That this was so was proved by the following experiment.

An enzyme preparation from mouse liver was divided into two equal portions (1) and (2). To (1) was added enough saccharate solution (pH 5.2) to bring the final concentration to 0.03 M with respect to saccharate. The enzyme was precipitated in both (1) and (2) by adding equal volumes of saturated ammonium sulphate solution at pH 5.2. The precipitated enzyme was dissolved in water and the activity measured. These enzyme solutions were again put through an ammonium sulphate fractionation as before. The precipitated enzyme was redissolved in water and the activity estimated again. Results of such an experiment are shown in Table (8).

Table (8)

Effect of Ammonium sulphate fractionation on saccharic acid "bound" to glucuronidase molecules

Treatment	* Hydrolysis (µg phenol)		% Recovery
	Fraction (1)	Fraction (2)	
First Am_2SO_4 fractionation 0-50% saturation	59	41	70
Second Am_2SO_4 fractionation 0-50% saturation	59	58	98

* Values are expressed per ml of initial enzyme preparation. Volumes of fractions (1) and (2) were kept equal at all stages.

From the results it is quite clear that the combination of inhibitor to enzyme is a very loose one. The 30% inhibition still observed after the first ammonium sulphate precipitation may be due to a slight adsorption of saccharate from the solution on to the precipitate thrown down by ammonium sulphate.

G) The effect of varying the substrate concentration on the inhibition caused by saccharate

The normal substrate activity curve for the hydrolysis of phenylglucuronide by mouse liver glucuronidase has been studied by Kerr et al. (1948, 1949). An approximate value of 0.0035 M was obtained for K_m - the

Michaelis-Menten constant. The value for K_m corresponds to the concentration of substrate at which half the maximum velocity of the reaction is reached. The maximum velocity was usually reached with 0.015 M substrate. Beyond this concentration of substrate a pronounced inhibitory effect on the hydrolysis was observed. These findings of Kerr *et al.* were confirmed.

From fig. (1) it will be seen that 50% inhibition of glucuronidase activity was caused by 2×10^{-4} M saccharate. The substrate concentration was 0.015 M. Under these conditions one could picture the active centres on the enzyme molecule as being equally divided between saccharate and phenylglucuronide molecules. The effect of keeping the substrate concentration fixed and altering the saccharate concentration was seen in fig. (1). Increasing the saccharate concentration resulted in a larger proportion of the active centres being occupied by saccharate molecules and thereby causing a greater percentage inhibition. A similar process would be expected to take place were the saccharate concentration kept fixed and the substrate concentration decreased. Fig. (2) shows the results of two such experiments. Hydrolysis was carried out in the presence of 2×10^{-4}M saccharate and varying concentrations of phenylglucuronide. The activity of the enzyme in presence of the inhibitor at each substrate concentration was expressed as a fraction of the activity of the enzyme in absence of inhibitor and in a substrate

concentration of 0.015 M. The fractional activities have been plotted against substrate concentration. The points on the graph show averages for two representative experiments one with liver glucuronidase fraction A and the other with fraction B. There was no appreciable difference in the results for the two fractions.

Fig. (2)

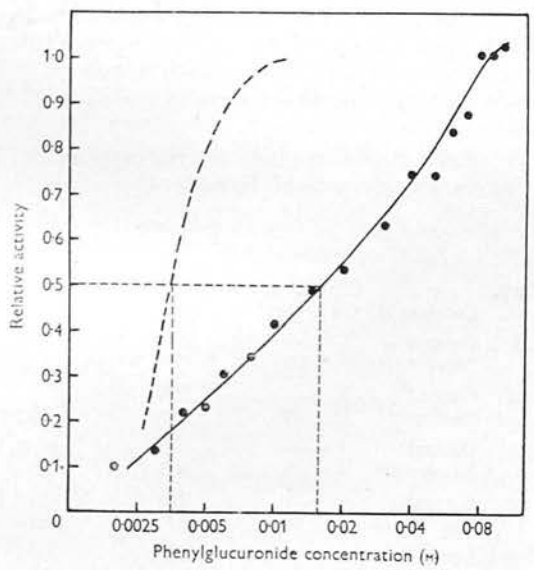


Fig. 2. Effect of varying concentrations of phenylglucuronide on its hydrolysis by mouse-liver glucuronidase in presence of 2×10^{-4} M-saccharate (●——●). Results expressed as fractions of the maximum activity observed in absence of inhibitor. Substrate-activity curve in absence of inhibitor (Kerr *et al.* 1948) shown by broken line.

It is clear from the nature of the curve that saccharate acted competitively since the inhibition decreased with increasing substrate concentration until at 0.08 M phenylglucuronide concentration, the activity of the enzyme was fully restored. The substrate concentration giving half the maximum velocity under these conditions is 0.015 M - as is to be expected - and is designated K'_m. (the apparent Michaelis Menten constant).

The dotted line shows a typical substrate optimum curve in the absence of inhibitor.

H) The dissociation constant K_I of the enzyme-inhibitor complex

The Michaelis-Menten constant, usually denoted K_m, gives an indication of the affinity of an enzyme for its substrate. By measuring the activity of an enzyme in varying concentrations of its substrate a value for K_m could be obtained (Michaelis & Menten 1913, Haldane 1930, Lineweaver & Burk, 1934). From a theoretical consideration of enzyme action one deduces that K_m is the substrate concentration at which half the maximum velocity of enzyme action is obtained.

In the presence of an inhibitor, the enzyme forms a complex with it and the dissociation constant of this inhibitor complex could be evaluated as shown below.

Consider the reaction

$$E + S \rightleftharpoons ES \longrightarrow E + \text{Reaction products}$$

Let e = concentration of enzyme

S = " " substrate

p = " " ES at any time

K_m = dissociation constant of enzyme substrate complex ES.

Applying the law of mass action

$$S(e-p) = K_m \cdot p \qquad \text{——————} \qquad (1)$$

or

$$p = \frac{e\,S}{K_m + S} \qquad \text{——————} \qquad (2)$$

Let v = velocity of decomposition of ES.

Then $v = kp = \frac{ke \quad S}{K_m + S}$ ——— (3)

Where k = constant

When S is large, p ⟶ e and the velocity of enzyme reaction would reach a maximum V.

Then $V = ke$ ——— (4)

and $v = \frac{V.\ S}{K_m + S}$ ——— (5)

<u>or</u> $K_m = S \left(\frac{V}{v} - 1 \right)$ ——— (6)

The value of K_m (dissociation constant, Michaelis-Menten constant) is given by the concentration of substrate giving rise to half the maximum velocity i.e. when $v = \frac{V}{2}$

Then $K_m = S \left(\frac{V}{V/2} - 1 \right)$

<u>or</u> $K_m = S$ ——— (7)

Consider a similar system but with an inhibitor now present. We then have

$$E + S \rightleftharpoons ES \longrightarrow E + \text{Reaction products}$$

$$E + I \rightleftharpoons EI$$

In addition to the previous symbols, let

I = concentration of inhibitor

q = concentration of inhibitor enzyme complex

K_I = dissociation constant of inhibitor enzyme complex

Applying the law of mass action

$S\ (e - p - q) = K_m\ p$ ——— (8)

$I\ (e - p - q) = K_I\ q$ ——— (9)

Eliminating q from equations (8) and (9),

$$p = \frac{e\,S}{K_m\left(1 + \frac{I}{K_I}\right) + S} \qquad \text{——} \quad (10)$$

Let v' be the velocity of reaction of this system.

Then

$$v' = \frac{k\,e\,S}{K_m\left(1 + \frac{I}{K_I}\right) + S} \qquad \text{——} \quad (11)$$

When S becomes large, $p \longrightarrow e$ and $v' \longrightarrow V$

Eq^{n} (11) then becomes

$$v' = \frac{V\,S}{K_m\left(1 + \frac{I}{K_I}\right) + S} \qquad \text{——} \quad (12)$$

$$\text{When } v' = \frac{V}{2}, \quad S = K_m\left(1 + \frac{I}{K_I}\right) \qquad \text{——} \quad (13)$$

This value of S may be designated K'_m - the apparent Michaelis-Menten constant in the presence of the inhibitor.

$$K'_m = K_m\left(1 + \frac{I}{K_I}\right) \qquad \text{——} \quad (14)$$

It is obvious from the equation that K'_m depends on I. From equation (14)

$$K_I = \frac{I \cdot K_m}{K'_m - K_m} \qquad \text{——} \quad (15)$$

and a value for the dissociation constant (K_I) of the inhibitor enzyme complex could be calculated if K_m and K'_m for a certain concentration of I are known

From Fig. (2) $K_m = .0035$ M

and $K'_m = .015$ M

when $I = 2 \times 10^{-4}$ M

Substituting these values in eq^{n} (15)

$$K_I = 6 \times 10^{-5} M$$

It should be stressed that presumably owing to the presence of impurities, K_m may vary from one enzyme preparation to another by as much as 50% in absence of added inhibitor. This variation is associated with variations in the substrate concentrations at which maximum activity is reached and inhibition by excess substrate becomes marked. The value for K_I would not be materially altered since K'_m varies with K_m and

$$K_I = \frac{K_m . I}{K'_m - K_m}$$

Hunter & Downs (1945) have evaluated K_i by a different procedure in the case of arginase. From eqns. (3) and (11), dividing one by the other

$$\frac{v'}{v} = \frac{K_m + S}{K_m + \frac{I.K_m}{K_i} + S} \qquad \text{——} \quad (16)$$

At a particular substrate and inhibitor concentration v' would correspond to the inhibited activity of the enzyme and v to the uninhibited activity. $\frac{v'}{v}$ would therefore represent the fractional activity and may be denoted by the term α.

Then $$\alpha = \frac{K_m + S}{K_m + I\,\frac{K_m}{K_i} + S} \qquad \text{——} \quad (17)$$

From eq^{n} (17)

$$I \left(\frac{\alpha}{1 - \alpha} \right) = \frac{K_i}{K_m} (K_m + S) \qquad \text{——} \quad (18)$$

$$\text{or } I\left(\frac{\alpha}{1-\alpha}\right) = K_i + \frac{K_i}{K_m} S \quad \text{——} \quad (19)$$

If at a fixed concentration of I, $\left(\frac{\alpha}{1-\alpha}\right)$ is measured at varying concentrations of S, then a graph of I $\left(\frac{\alpha}{1-\alpha}\right)$ against S should give a straight line. The intercept would represent K_i. Hunter & Downs used this equation quite successfully with arginase and various inhibitors. When attempts were made to evaluate K_i for saccharic acid and glucuronidase by this method, the results obtained could not be made to fit a straight line graph. The points were very widely scattered. It is, however, possible that if several estimations are made at each point and the average plotted as was done by Hunter & Downs, a suitable graph may be obtained from which K_i and K_m may be calculated.

Table (9) shows the results of one of the experiments, carried out for this purpose, as an illustration of the difficulty encountered.

TABLE (9)

Ratio of inhibited activity to inhibition at various substrate concentrations in the presence of 2×10^{-4} M saccharic acid.

Subst. Conc^{n} ($\times 10^{-3}$ M)	Hydrolysis μg phenol liberated		$\frac{\alpha}{1-\alpha}$
	In presence of inhibitor (α)	In absence of inhibitor (1)	
4	8.1	23.0	.55
6	10.4	28.0	.59
8	16.0	30.7	1.09
10	16.5	34.0	.94
12	17.3	35.9	.93

I) The in vivo effect of saccharic acid on growth and tissue glucuronidase activity in the mouse

A compound that inhibits β-glucuronidase activity in vitro can hardly be expected to arrest any mechanism, responsible for the increase in the activity of the enzyme normally observed in vivo, when a tissue is stimulated to growth . If, however, glucuronidase plays an essential part at some stage in the growth process, then administration of an inhibitor would upset the normal process at that stage. This interference would then probably manifest itself either in some form of abnormality or in the death of the animal.

A number of experiments were carried out with saccharic acid to test this possibility . When large doses were administered to mice, saccharic acid was apparently without effect on growth in infant mice or on liver regeneration in adults after partial hepatectomy. Figures for glucuronidase activity, the weights of single organs and the whole animals, and the histological picture were invariably identical with those observed in the appropriate controls. During the preparation of the enzyme for assay any saccharic acid initially present in the tissue would be eliminated.

The experiments are described very briefly.

Experiment (1)

Three adult mice were injected intraperitoneally with a 1.5% (w/v) solution of potassium-hydrogen-

saccharate in 0.85% saline adjusted to pH 7.0. Injections were given at six-hour intervals, the dose administered at each injection being 100 mgm/Kgm body weight, 36 hours after the first injection and 6 hours after the last one, the animals were killed. All three animals looked quite fit and normal at the end of the experiment. Two of the livers looked slightly spotted. Glucuronidase activities of liver and kidney were normal.

Experiment (2)

Litter mates, fourteen days old and weighing from 8-9 gms each were separated into two groups of 4 animals each. The mice in one group were injected intraperitoneally with a 1.5% (w/v) solution of potassium-hydrogensaccharate in 0.85% saline adjusted to pH 7.0, three times a day - each dose administered being 300 mgm/Kgm body weight. The animals in the other group were injected an equal volume of 0.85% saline. At the end of 6 days the animals were killed, and the glucuronidase activities of the liver and kidney estimated. There was no apparent difference in the glucuronidase activities between the two groups. Histological examination of the liver and kidney by Dr. J. G. Campbell showed no abnormalities. The average weight of the animal in the two groups increased by the same amount - 3 gm/animal - over the 6 days.

Experiment (3)

Mice from 3 litters - two weeks old and weighing about 9-10 gms each were separated into three groups of six mice - each litter being equally divided between the three groups. The animals in the first group were injected subcutaneously (2 gm/Kgm body weight) with a 10% (w/v) solution of potassium-hydrogen-saccharate in 0.85% saline adjusted to pH 7.0, thrice daily for 3 weeks. Those in the second group were injected intraperitoneally with a similar dose. Those in the third group were used as controls.

The animals were weighed daily. The increase in weight per mouse over the three weeks was almost the same - 10 gms. - in all three groups.

Experiment (4)

In this experiment, the potassium-hydrogen-saccharate was incorporated in the food fed to the animals. A number of young mice weighing around 9 gms. each and approximately the same age were divided into two groups. One group was fed a mixture of:-

Rat cake ..... 250 gms.

Bread crumbs ..... 150 gms.

K-H-Saccharate ... 12 gms.

The other group was fed a similar mixture but with the K-H-Saccharate replaced by 9 gms. of glucose and 3.6 gms. potassium chloride.

The animals in both groups had access to plenty of food. The animals were weighed daily. The

average increase in weight per mouse over a period of 10 days was the same (7 gms.) in both groups.

Experiment (5) (This was done in conjunction with Dr. Kerr.)

Ovariectomized mice were separated into 3 groups. The animals in all groups were injected intraperitoneally (2.5 gm/Kgm body weight) with a 10% (w/v) solution of K-H-saccharate in 0.85% saline adjusted to pH 7.0, thrice daily. On the second day the animals in one group underwent partial hepatectomy, while those in another were given an injection of carbon tetrachloride in olive oil (5.3 g/Kgm body weight). After a suitable time had elapsed, the animals were killed, and the glucuronidase activities of the liver, kidney and uterus were estimated. The uterine weights were also noted. Results are shown in table (10).

The glucuronidase activities were not depressed in any of the tissues examined. The activities of liver, kidney and uterus in the three groups of animals were not different from those in the corresponding animals to which saccharate had not been administered.

Partial hepatectomy causes a rise in the glucuronidase activity of liver and uterus in ovariectomized mice. This is noticeable even after 8 days. The uterine weight also increases (Kerr et al 1949). The administration of saccharate did not prevent this rise.

Carbon tetrachloride produces effects similar to those observed after partial hepatectomy. The administration of saccharate did not prevent the rise in the ovariectomized animals which had been administered carbon tetrachloride.

TABLE (10)

Effect of injected saccharic acid on the glucuronidase activity of liver, kidney and uterus in castrated* female mice

Treatment	Examined after (days)	Sex	G.U. /g. moist tissue			Uterine weight (mg)
			Liver	Kidney	Uterus	
¥ None	-	F	334 ± 48 (6)	266 ± 39 (6)	335 ± 53 (5)	234 ± 55 (6)
		CF	250 ± 49 (6)	261 ± 43 (6)	174 ± 45 (6)	34 ± 18 (6)
¥ Partial Hepatectomy	8	CF	535 ± 61 (6)	328 ± 54 (6)	395 ± 43 (6)	115 ± 19 (6)
¥ Carbon Tetrachloride (5.3 g/kg)	7	CF	715 ± 60 (6)	290 ± 59 (6)	496 ± 64 (6)	77 ± 23 (6)
Saccharate (2.5 g/kg) Thrice daily	4	CF	258 ± 10 (3)	390 ± 20 (3)	184 ± 24 (3)	36 ± 12 (3)
Saccharate (as above) + partial hepatectomy on 2nd day	10	CF	573 ± 56 (3)	353 ± 27 (3)	344 ± 34 (3)	104 ± 16 (3)
Saccharate (as above) + carbon tetrachloride (5.3 g/kg) on 2nd day	9	CF	758 ± 49 (3)	359 ± 40 (3)	337 ± 45 (3)	111 ± 19 (3)

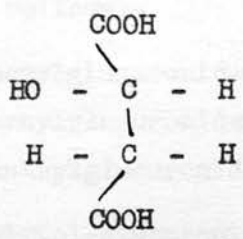
¥ Quoted from Kerr et al (1949)

* ovariectomized

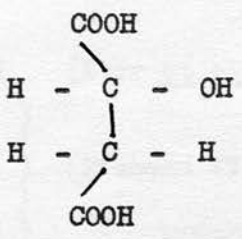
DISCUSSION

The inhibition of glucuronidase activity by saccharic acid is evidently competitive and reversible . This is not surprising considering the structural similarity of saccharic acid to glucuronic acid. Changing the carboxyl group at C_6 in saccharic acid to a primary alcohol group to give gluconic acid or changing the configuration to give mucic acid resulted in a considerable diminution of inhibitory power. The effect of glucuronic acid on the hydrolysis of phenylglucuronide by the enzyme may have been inhibition in the usual sense or a mass action effect. Hydrolysis of a glucuronide by glucuronidase is known to result in the formation of free glucuronic acid (Levvy, 1948).

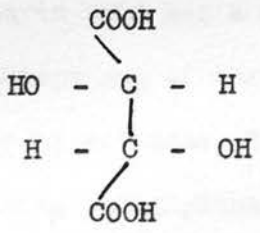
While L(-)-malic acid causes inhibition of enzyme activity D(+)-malic acid appears to be without any action judging by the results obtained with DL-malic acid. The tartaric acid corresponding to D(+)-malic acid, viz. L(+)-tartaric acid, was without effect on glucuronidase activity. D(-)-tartaric acid was not available for study. Oshima (1936) has observed that the configuration of the β-hydroxy group may be the factor responsible for the inhibitory action of hydroxy acids. See structural formulae shown below.



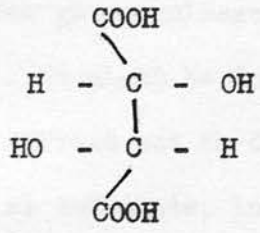
L (-) Malic acid



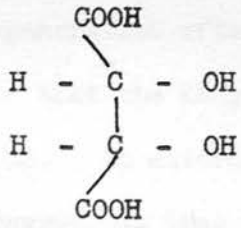
D (+) Malic acid



D (-) Tartaric acid



L (+) Tartaric acid



Meso -Tartaric acid

A comparative study of the D- and L-tartaric acids on glucuronidase activity would probably throw some light on this aspect of the problem.

Considerable difficulties were encountered in determining K_i - the dissociation constant for the inhibitor enzyme complex - in the case of saccharic acid and β-glucuronidase. It is considered, however, that the value of 6×10^{-5}M finally arrived at is at least as reliable as values quoted for K_m - the dissociation constant of the substrate enzyme complex, in the hydrolysis of biosynthetic glucuronides by β-glucuronidase. Figures available for K_m are as follows:-

Phenylglucuronide	0.0035 M	(Kerr et al 1948)
Bornylglucuronide	0.01 M	(Fishman 1939.)
Menthylglucuronide	0.004 M	
Estriol-glucuronide	0.0005 M	
Phenolphthalein-glucuronide	0.00005 M	(Talalay et al 1946.)

Saccharic acid has a higher affinity for glucuronidase than all except one of these glucuronides. It might be interesting to repeat some of the experiments carried out to determine K_i, using phenolphthalein glucuronide as substrate, instead of phenol glucuronide.

The failure of saccharic acid in large doses to modify liver regeneration after damage or growth in infant mice, may indicate that the enzyme is not directly concerned in cell division. An alternative and highly probable explanation, however, is that saccharic acid is too rapidly metabolised or excreted to produce any perceptible changes *in vivo*. It is also possible that normal cell division may involve the hydrolysis of a naturally occurring glucuronide with a much greater affinity than that of saccharic acid.

Carr (1947) has shown that the prolonged administration of saccharic acid to rats for several generations caused no change in the structure of any tissue examined or in the growth of the animals. It is very likely that as saccharic acid resembles glucose it is very rapidly metabolised in the body.

Kerr *et al*, in this laboratory, have observed that saccharate has a protective action on mouse liver when administered along with menthylglucuronide. Histological examination showed little damage in the liver cells. Menthylglucuronide, when administered alone causes liver damage (Levvy *et al* 1948). A similar protective

effect was obtained with glucose in equivalent amounts. Saccharate probably acts as a source of carbohydrate and maintains and protects the liver.

The use of a substituted saccharic acid which is not easily metabolised should be of considerable help in in vivo studies.

SECTION (ii)

THE GLUCURONIDE-SYNTHESIZING SYSTEM IN THE MOUSE AND ITS RELATIONSHIP TO β-GLUCURONIDASE

INTRODUCTION

The excretion of glucuronides in the urine of animals under certain conditions has been known for a long time. The formation of glucuronides was at one time regarded as one of the chief detoxication mechanisms that have been evolved by the animal body to rid itself of toxic substances, both of endogenous and exogenous origin. It is probably safer to say that it represents one stage in a chain of imperfectly understood metabolic processes.

Chemically d-glucuronic acid is an oxidation product of d-glucose and some of the earlier workers assumed that in vivo glucuronic acid was produced by the direct oxidation of glucose.

Schmiedeberg and Meyer (1879) maintained that the formation of conjugated glucuronides in the animal body occurred through the oxidation of glucose to glucuronic acid and subsequent condensation of the glucuronic acid molecule with a suitable aglucone. Sundvik (1886) and Fischer & Piloty (1891) on the other hand held the view that a glucoside was first formed in the animal body and then oxidized to the corresponding glucuronide.

The experiments of Hildebrandt (1905, 1909) appeared to support the latter hypothesis. On subcutaneous injection of coniferin (coniferyl glucoside), syringin (methoxy coniferyl glucoside) or bornylglucoside to rabbits, the corresponding conjugated glucuronic acids were excreted

in the urine. Two explanations are possible. Either the glucoside was directly oxidized to the glucuronide or the glucoside was hydrolysed to give the free aglucone which then combined directly with preformed glucuronic acid or its precursors.

Schüller (1911) observed that the glucoside phloridzin when administered to animals was excreted as phloridzin glucuronide. The original glucose molecule remained intact while the glucuronic acid was attached to one of the originally free hydroxyl groups of phloridzin. In this case glucuronic acid was not obtained from the glucose of phloridzin.

Pryde & Williams (1936) showed that when phenyl-β-d-glucoside was injected into or fed to rabbits, there was increased excretion of ethereal sulphate in the urine equal to that produced by an equivalent amount of free phenol. This indicated the in vivo hydrolysis of phenyl-β-d-glucoside to phenol.

In a pump-lung-liver-kidney perfusion set up, it was observed by Hemingway et al (1934) that while borneol or phenol when added to the perfusing blood were converted into the corresponding glucuronides, none was formed on the addition of the glucosides.

Lipschitz & Bueding (1939) have shown that guinea pig liver slices produce conjugated glucuronic acids in the presence of suitable concentrations of borneol but do not produce glucuronide from bornyl-β-glucoside.

The experiments of Schüller, Pryde & Williams, Hemingway et al , and Lipschitz & Bueding effectively dispose of the Sundvik, Fischer & Piloty hypothesis that a glucoside is directly oxidized to a glucuronide in vivo.

Proteins, glycogenic amino-acids, glycogen and 3-carbon metabolites of carbohydrate metabolism have been suggested as sources of glucuronic acid.

In the earlier work, starved animals (dogs and rabbits) were fed glucuronidogenic substances and the excretion of conjugated glucuronides in the urine studied. The experiments of Thierfelder (1886) and Mandel & Jackson (1903) suggested that protein was the source of glucuronic acid. Experiments with starved animals were unsatisfactory as there was no way of being quite sure that the animal was free from glycogen.

Schmid (1936) to overcome this difficulty worked with hibernating frogs. During hibernation the total glycogen content is of the order of 0.25 mg whereas a well fed frog has 60 - 200 mg. of glycogen in its liver. He showed that preformed carbohydrates were necessary for the conjugation of glucuronic acid.

Quick (1926), working with depancreatized and normal dogs, concluded that the precursor of glucuronic acid was derived more readily from glycogen and glycogenic amino acids than from glucose. The possibility of a simple compound such as lactic acid - which in a diabetic animal is derived from protein and in a normal animal from the degradation of glucose -

being the source of glucuronic acid was suggested by Quick. This suggestion has received considerable support from the tissue slice experiments of Lipschitz & Bueding (1939). They showed that surviving liver slices were capable of synthesizing glucuronides *in vitro* , when incubated with a suitable aglucone in a synthetic medium. The production of conjugated glucuronides by liver slices from fasting animals was stimulated by certain 3-carbon compounds like pyruvic acid, dihydroxyacetone and lactic acid, while glucuronic acid itself or glucosides, maltosides, glucose, other carbohydrates and biologically important carbohydrate metabolites such as glyceraldehyde, phosphoglycerate, methylglyoxal, α-glycerophosphate and hexosediphosphate did not stimulate glucuronide formation. An oxidative process catalysed by a heavy metal was considered necessary for supplying the energy required for the synthesis. Glucuronic acid production was sensitive to iodoacetate and fluoride suggesting that a phosphorylation mechanism was involved in the process.

With the discovery of β-glucuronidase which catalyses the theoretically reversible reaction

$$\beta\text{-glucuronide} \rightleftharpoons \text{aglucone} + \text{glucuronic acid}$$

the question of whether glucuronic acid can combine with a hydroxy compound to form a β-glucuronide needs more careful investigation.

Some hydrolytic enzymes - notably the esterases - have been shown to catalyse the reverse process *in vitro* under conditions which were highly "unphysiological".

There are, however, several instances where two entirely different enzyme systems are required to catalyse the reaction in the forward and backward directions respectively. Urease catalyses the reaction, Urea ⟶ carbon-dioxide and ammonia. In the animal body, however, urea is produced from arginine by the action of arginase. While starch is hydrolysed to maltose by amylase, the synthesis of starch evidently goes by way of a dephosphorylation of glucose-1-phosphate catalysed by the enzyme glucosan phosphorylase. The breakdown of acetylcholine to acetic acid and choline is catalysed by cholinesterase while its synthesis *in vivo* is brought about by choline acetylase.

It would therefore appear that while the fact that β-glucuronidase breaks down β-glucuronides to an aglucone and glucuronic acid is well established, hardly any evidence is available to suggest that it catalyses the synthesis of glucuronides *in vivo*.

Florkin *et al.* (1942) claim to have effected the conjugation of borneol with glucuronic acid in the presence of ox spleen glucuronidase. The evidence is, however, based on measurements of small differences. The work of De Meio & Arnolt (1944) also suggest that phenol may combine directly with glucuronic acid in liver slices. They found that the inhibition of glucuronide conjugation caused by iodoacetate could be reversed by the addition of glucuronate while lactate was without effect. Glucuronic acid, however, did not re-establish conjugation after inhibition by cyanide or

anaerobiosis. The inhibitory effect of iodoacetate would appear from the data to affect the formation of glucuronic acid and not its conjugation with phenol. Since, however, their method does not appear to measure glucuronide formation (see Levvy & Storey 1949) their results are open to doubt and criticism.

Fishman (1940) has postulated a synthetic role for β-glucuronidase *in vivo*. This hypothesis was mainly based on the evidence of increased glucuronidase activity in the liver and uterus of animals following the administration of glucuronidogenic substances. The increase in enzyme activity was considered an adaptation process. The work of Levvy *et al.* (1948, 1949) in this laboratory has cast doubt on Fishman's hypothesis. They suggest that increased enzyme activity in tissues is associated with increased proliferative activity caused by various measures which induced tissue damage followed by repair processes *or* stimulated cell proliferation as in partial hepatectomy. The agents used by Fishman were shown to cause proliferative changes in liver and uterus. Furthermore, the substances used in the experiments of Levvy *et al.* to cause tissue damage were not all glucuronidogenic. Fishman's assumption that glucuronidase acts synthetically *in vivo* is thus no longer necessary to explain his experimental findings.

Experiments have been conducted to find out if the enzyme system catalysing the synthesis of glucuronides *in vivo* was identical with or quite different from β-glucuronidase. The distribution of the glucuronide synthesizing system

in the mouse has been studied and its development in the liver with the age of the mouse followed. Substances which, when administered to the mouse, produce a marked effect on liver glucuronidase activity have been examined for their effect on the glucuronide synthesizing system in the liver under similar conditions. Certain other substances, including saccharic acid have been studied for their in vitro effect on the synthesis of o-aminophenylglucuronide by liver slices from normal mice. Finally, the synthesis of o-aminophenylglucuronide by glucuronidase preparations has been attempted.

The results of all the experiments carried out suggest very strongly that the glucuronide synthesizing system is quite distinct from β-glucuronidase.

EXPERIMENTAL

Measurement of glucuronide synthesis

The conversion of o-aminophenol to its glucuronide by tissue slices in sulphate free bicarbonate Ringer solution was followed by the method of Levvy & Storey (1949). The composition of the bicarbonate Ringer solution was as follows:-

Sodium bicarbonate (gassed with CO_2)	1.54 M	42.0 mls.
Sodium chloride	1.54 M	20.0 "
Potassium chloride	0.62 M	2.0 "
Magnesium chloride. $6 H_2O$	0.31 M	1.0 "
Pot. dihydrogen phosphate	0.154 M	2.0 "
Water	——	185.0 "
Calcium chloride	0.22 M	3.0 "

The solutions were mixed together in this order and to this basal Ringer medium were added 5.0 mls. M sodium lactate pH 7.4, 52.0 mg. ascorbic acid and 6.5 mg. o-aminophenol. After the solids had dissolved, the solution was cooled in ice and a brisk current of 5% CO_2 in O_2 passed through it for 10 minutes.

Preparation of tissue slices

The animal was killed by a blow on the head and the required organ dissected out as rapidly as possible and transferred to the ice-cold Ringer solution in a Petri dish. Pieces of the organ were held between two ground glass slides and slices about 0.4 mm. in thickness were cut by means of a safety razor blade according to a modified method of Deutsch (1936) - (see Umbreit et al.1945, p.75). The outermost slices were discarded. In experiments with lung, the intact lobes were used. Boyland & McDonald (1948) have shown that this is permissible for measurements of metabolism in lung from young adult mice. With very young animals it was necessary to pool

the slices from the organs of several animals.

Procedure

The tissue slices were incubated in the bicarbonate Ringer medium, prepared as described above - in an atmosphere of 5% CO_2 in O_2 for an hour at 37°C. At the end of the incubation slices were removed for dry weight estimations. Any soluble protein in the medium was precipitated by the addition of trichloroacetate-phosphate mixed buffer pH 2.25. After centrifuging down the precipitated protein, an aliquot of the clear supernatant was diazotized and coupled with naphthyl-ethylenediamine, when the o-aminophenylglucuronide formed gave a pink colour. This colour was estimated colorimetrically with a Spekker Absorptiometer using Ilford Filter No. 605. (Spectrum Yellow-green).

Enough tissue slices to give not less than 10 mg. dry weight (at 110°C) were used for each flask. Estimations were done in quadruplicate for each animal to reduce the variable error in the procedure. Control flasks with liver slices in Ringer medium not containing o-aminophenol were also set up in the earlier experiments. No pink colour was observed in these controls and in subsequent experiments these controls were omitted.

Results are expressed in the tables as µg o-aminophenol conjugated per gm. dry weight of the tissue in 1 hr.

Measurement of glucuronidase activity

The glucuronidase activity in tissue extracts was determined mainly by the procedure of Kerr et al. using phenyl-glucuronide as the substrate. The procedure has been briefly described in Section (1).

In some experiments where this substrate proved unsatisfactory, the experiments were later repeated using phenolphthalein glucuronide as substrate (Talalay et al. 1946). The original procedure was slightly modified to meet our requirements (See Section iii p. 84.).

The procedure finally adopted was as follows:- 0.5 ml enzyme, 0.5 ml substrate and 3.0 ml 0.1 M buffer pH 4.5 or 5.2 were incubated together for an hour at 37°C. At the end of the incubation, the colour of the phenolphthalein liberated was developed with 0.25 M $Na_2 CO_3$ - 0.4 M glycine mixed buffer. The addition of the carbonate-glycine buffer stops the enzyme action at the same time. The colour was read on a Spekker Absorptiometer as in the previous method, using an Ilford filter No. 605 (Spectrum yellow green). Results are expressed as µg phenolphthalein liberated per gm wet weight of tissue per hour at 37°C unless otherwise stated.

RESULTS

A) Glucuronide synthesizing power of various mouse tissues

The glucuronide synthesizing power of liver, kidney, spleen and lungs in young and adult mice and also of two transplantable tumours in the mouse has been studied. Table (11) shows the results obtained with these tissues.

TABLE (11)

The glucuronide-synthesizing power and β-glucuronidase activity of various mouse tissues

Tissue	Age of animal	o-Aminophenol conjugated (μg./g. dry weight)	Glucuronidase activity/g. moist weight (G.U.)
Liver	Adult	570 ± 43 (41)	273 ± 13 (23) ¥
Kidney	Adult	150 ± 14 (4)	363 ± 24 (11) ¥
	9 days	30 (4)	—
	5 days	—	793 (4) ¥
Lung	Adult	Nil (2)	185 ± 19 (3)
	9 days	Nil (3)	316 ± 22 (3)
Spleen	Adult	Nil (3)	636 ± 70 (23) ¥
	9 days	Nil (4)	—
	5 days	—	3245 (4) ¥
Sarcoma (Crocker 180)	Adult	Nil (2)	433 ± 44 (6)
Carcinoma (Imperial Cancer Research Fund 2146)	Adult	Nil (2)	751 ± 75 (6)

¥ Quoted from Levvy et al. 1948.

(When the mean is based on values for individual animals the standard error of the mean for the group is also shown. Figures in brackets are numbers of animals used.)

Mice about 6 weeks of age and over and weighing 25 gms. or more were treated as adults. They were drawn from 3 different colonies. The glucuronide synthesizing power of adult mouse liver did not appear to vary from one strain to another. Differences in sex did not show any apparent effect on the synthesizing activity and hence no differentiation of sex has been made in these and later experiments.

In agreement with Lipschitz & Bueding (1939) the glucuronide synthesizing system was found only in liver and kidney of the tissues examined. The latter was much the less active of the two tissues. The ability of liver and kidney slices from young mice to synthesize glucuronides was considerably less than that of slices from adult animals. Lung and spleen showed no synthetic activity, neither did the two transplantable tumours that have been examined.

Also included in table (11) are figures for the glucuronidase activities of the various tissues - some quoted from Levvy et al (1948), while others were specially determined for the purpose by Dr. L.M.H. Kerr (Karunairatnam et al 1949). The higher glucuronidase activities of organs from young mice compared with those from normal adults, already noted for liver, kidney, spleen and uterus (Levvy et al 1948; Kerr et al 1949) is also seen in lung.

The distribution of the enzyme β-glucuronidase obviously bears no relation to that of the synthetic system.

B) The development of the glucuronide synthesizing system in mouse liver

Since the glururonidase activity of young mouse liver has been found to be very high and its glucuronide synthesizing power very low compared to values for adult mouse liver, the development of the glucuronide synthesizing system in the liver with the age of the mouse was studied. Mice from our own mixed colony were used in this experiment. Table (12) shows the synthetic power of the liver at the various ages. These results are expressed graphically in Fig. (3).

TABLE (12)

The glucuronide synthesizing activity of the liver at varying ages of the mouse

Age of animal (days)	No. of animals examined	o-aminophenol conjugated (µg/g. dry weight)	Remarks
2	4	0	Slices pooled
3	9	40	" "
5	11	60	Two batches of pooled slices
7	8	30	" "
10	6	110	" "
13	4	180	" "
17	6	300	Three batches of pooled slices
20	6	420	" "
23	7	380	Individual animals
25	4	550	" "
28	11	490	" "
31	3	600	" "
35	4	550	" "

Fig. (3)

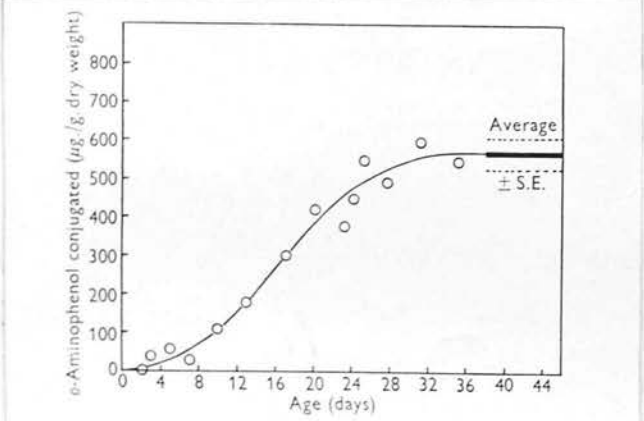


Fig. 1. The development of the glucuronide-synthesizing system, as measured by the conjugation of *o*-aminophenol, in the liver of the growing mouse (own mixed colony).

With very young mice, the liver slices were pooled from a number of animals to give enough slices for a quadruplicate determination.

The change in the liver glucuronidase activity with the age of the animal has been fully studied from birth until maturity by Dr. L.M.H. Kerr in this laboratory. The results of this study, expressed graphically in Fig. (4) have been included to help in following the discussion.

Fig. (4)

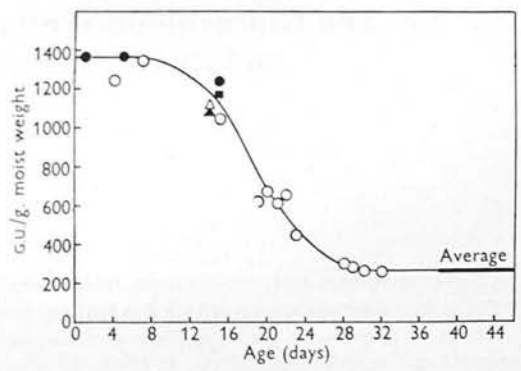


Fig. 2. The change in glucuronidase activity in the liver of the growing mouse: ■, strain C57; △, strain A; ▲, strain CBA; ●, own mixed colony, August 1947; ○, own mixed colony, February 1949.

The synthesizing power of liver starts at zero at birth and gradually develops as the mouse grows up reaching steady adult values between 4 and 5 weeks of age. The glucuronidase activity on the other hand starts at a high value at birth and gradually falls to adult values about the same time. If β-glucuronidase was the enzyme concerned in the synthesis of glucuronides, one would expect the synthetic activity of the liver from young mice to be considerably higher than that of adult mice. That this is not the case suggests that the glucuronide hydrolysing enzyme and the glucuronide synthesizing enzyme(s) are two distinct enzyme systems.

C) <u>The influence of β-glucuronidase on glucuronide synthesis</u>

The possibility had to be considered that the activity of the glucuronide synthesizing system in the liver did not vary with age, but that the hydrolytic activity of the liver glucuronidase at any given age determined the net amount of synthesis which can be measured - compare Figs. (3) and (4).

Saccharic acid causes almost complete inhibition of glucuronidase activity in a concentration of 10^{-2} M. The effect of saccharic acid, in this concentration, on the synthetic power of mouse liver and other organs was therefore studied to settle this point.

In making up the bicarbonate Ringer solution, a concentrated solution of saccharic acid adjusted

to pH 7.4 was added in place of part of the water to bring the final concentration of saccharate in the Ringer solution to 10^{-2} M. In the control flasks, however, the saccharate was omitted in making up the Ringer solution.

With a tissue which had a high glucuronidase activity and low synthetic power, it was thought that the addition of saccharic acid to the Ringer medium might result in a higher synthetic activity. Consequently, some of the experiments with liver slices from young mice and kidneys, lung, spleen and tumour tissues from adult mice were repeated with and without saccharic acid in the bicarbonate Ringer medium. The results of these experiments are given in the next table.

TABLE (13)

The effect of 10^{-2} M saccharic acid on glucuronide synthesis by various organs of the mouse

Organ	Age of mouse (days)	µg o-aminophenol conjugated per g. dry weight	
		In controls	In presence of saccharic acid
Liver	6	80	60
	17	300	210
Kidney	Adult	150	50
Lung	"	0	0
Spleen	"	0	0
Sarcoma (Crocker 180)	"	0	0
Carcinoma (Imperial Cancer Research Fund 2146)	"	0	0

It can be seen that saccharic acid caused no increase in the synthetic power of infant liver or adult kidney; nor did its presence lead to glucuronide synthesis by lung, spleen or tumour tissues. The slight decrease noted in the synthetic power of liver slices from young mice would probably be due to the non-specific effect of the dicarboxylic acid as shown by Dr. Levvy in the experiments with adult liver slices (Karunairatnam, [redacted] & Levvy, 1949).

Before one concludes from the results of these experiments that the glucuronide synthesizing system is absent in mouse lung, spleen and tumours, small in kidney and that the synthesizing system in the liver develops with age, two further questions have to be answered.

1) Does saccharic acid inhibit the hydrolysis of o-aminophenylglucuronide?

2) Does saccharic acid penetrate tissue slices?

The answer to the first question is provided by the results of experiments carried out by Dr. G. A. Levvy which show that saccharic acid does inhibit the hydrolysis of o-aminophenylglucuronide by mouse liver glucuronidase (cf. Karunairatnam & Levvy, 1949). With a 0.13% solution of o-aminophenyl-glucuronide 11% hydrolysis was obtained in absence of saccharate and 0.7% in the presence of 10^{-3} M saccharate. In another experiment the corresponding values were 17% and 3.6%.

Similar experiments using liver slices in place of glucuronidase preparations have been carried out by the author. In the method employed to measure hydrolysis, disappearance of o-aminophenylglucuronide was estimated after an hour's incubation of tissue slices in a 0.14% solution of o-aminophenylglucuronide. Although this method is quite satisfactory when using glucuronidase preparations as in the experiments of Dr. G. A. Levvy, it was not reliable when using tissue slices. The results obtained, however, show that hydrolysis of o-aminophenylglucuronide by tissue slices does take place and that this hydrolysis is inhibited by saccharic acid.

D) Penetration of saccharic acid into the cell

Mouse liver slices of known weight from two animals were shaken in sulphate-free bicarbonate Ringer solution containing 0.01 M saccharic acid for 90 minutes at 37°C. At the end of this period, the slices were removed, washed in three changes of distilled water and homogenized. Inactive protein was removed by incubating the homogenate at pH 5.2 at 37°C for 30 minutes and subsequent centrifugation. Without further purification, the supernatant was examined for β-glucuronidase activity. The activity in terms of μg phenol liberated at 37°C in 1 hr. by a gm. of liver was 220 compared with 334 for control slices from the same two animals put through the identical procedure in absence of saccharic acid. Unless saccharic acid was so strongly adsorbed on the surface of the slices that three washings with distilled water had not

removed it, it would appear from the results that an appreciable amount of saccharate had penetrated the cells. On the assumption that saccharate concentration within the slices rose to 0.01 M, the inhibition expected would have been of the order of 75%. The percentage inhibition actually observed was 33%.

In other experiments, the enzyme preparation in the assay procedure for glucuronidase activity was replaced by mouse liver slices of known weight. At the end of the incubation period, the slices were removed and their dry weights (110°C) determined. When phenylglucuronide was used as substrate in the assay procedure, the enzyme blanks were found to be high and variable. The results had therefore little quantitative value, but nevertheless they suggested that some hydrolysis of phenylglucuronide took place and that this process was strongly inhibited by 0.015 M saccharate.

When the modified phenolphthalein glucuronide method was brought into use in this laboratory, this experiment was repeated. Tissue slices of known weight were incubated with acetate buffer, pH 5.2, in the presence of the inhibitor solution at 37°C for an hour. The inhibitor solutions were adjusted to pH 5.2 (glass electrode) before being added to the incubation tubes. At the end of the hour's incubation, the colour of the phenolphthalein liberated was developed by the addition of Na_2CO_3 - glycine buffer. Slices were removed, the solutions centrifuged and the colours of the clear supernatants estimated. The results are expressed in Table (14).

TABLE (14)

The effect of saccharic acid and sodium fluoride on the hydrolysis of phenolphthalein glucuronide by mouse liver slices

Inhibitor	Conc^{n} (M)	µg phenolphthalein liberated /g moist tissue	% Inhibition
None	-	1150	-
Saccharic acid	.01	0	100
Sodium fluoride	.015	900	22

The enzyme blanks were zero, suggesting that very little protein had escaped into the surrounding medium from the tissue slices. Further, the slices in the tubes showing hydrolysis were observed to be faintly pink even after washing them several times in water. The results suggest that the hydrolysis of phenolphthalein glucuronide by liver slices is inhibited very strongly by saccharic acid (0.01 M). The low enzyme blanks and the faint pink colour in the washed slices from the tubes showing hydrolysis suggest that the hydrolysis of phenolphthalein glucuronide had taken place within the cells and not so much in the buffer medium by enzyme diffusing out of the cells.

Lipschitz & Bueding have observed that sodium fluoride in a concentration of 0.015 M caused almost complete inhibition of glucuronide formation by guinea pig liver slices. It is interesting to note that this concentration of sodium fluoride causes only about 22% inhibition

of hydrolysis by liver slices. It has no inhibitory action, however, on liver glucuronidase preparations (see previous section).

Although the preceding experiments all suggest that saccharate does penetrate the intact cells, it was felt that more direct evidence on the point would be welcome. It was therefore suggested to Dr. J. G. Campbell, that he should study the effect of saccharic acid on the two histochemical tests developed by Friedenwald & Becker for β-glucuronidase (1948). Campbell (1950) has shown that in the presence of saccharic acid of varying concentrations, the typical colour formed at the presumed site of glucuronidase activity in mouse liver slices, was developed very slowly or not at all. In some experiments thick blocks of tissue were incubated with substrate, with and without saccharic acid, and sections, cut from the deeper layers of the tissue, examined. His experiments prove quite satisfactorily that saccharic acid does penetrate intact cells thereby confirming our own inferences from not very adequate information.

E) <u>The effect of saccharate on the oxygen uptake and anaerobic glycolysis of mouse liver slices</u>

Saccharate in a concentration of 0.014 M had no effect either on the oxygen uptake or on the anaerobic glycolysis of mouse liver slices as measured in the Warburg apparatus. The Ringer solutions of Krebs & Henseleit (1932) were used. Oxygen uptake was determined in phosphate

Ringer and an atmosphere of O_2, while the carbon dioxide output was measured in bicarbonate Ringer and an atmosphere of 5% CO_2 in N_2.

Table (15) gives the results of these experiments.

TABLE (15)

The effect of saccharic acid on the aerobic and anaerobic respiration of liver slices

Aerobic	Liver slices from normal animals	Q_{O_2} *	In glucose	5.09
			In saccharic acid	4.52
			In water	5.44
Anaerobic	Liver slices from animal starved for 18 hours	Q_{CO_2} *	In glucose	5.35
			In saccharic acid	4.23
			In water	4.99

* Expressed as μl O_2 or CO_2 per mg. dried tissue per hour.

The differences in Q_{O_2} and Q_{CO_2} were about 20% and these were not considered significant. There is the possibility that the slight depression of Q_{O2} or Q_{CO2} noted in the presence of saccharic acid might account for the slight depression of the synthetic activity observed when normal adult liver slices were incubated in the presence of saccharic acid.

F) The effect of various measures on the glucuronide synthesizing system of liver in vivo

Levvy et al (1948) and Kerr et al (1949) showed that injecting certain compounds into a mouse or carrying out partial hepatectomy on the mouse resulted in a rise in the glucuronidase activity of liver and other organs to two or three times its normal activity. The glucuronide synthesizing system of the mouse liver has been examined after the mouse had been subjected to such measures and at the times corresponding to a marked change in glucuronidase activity. Table (16) summarizes the results of these experiments. The figures for glucuronidase activity are quoted from Levvy et al (1948) to help in following the discussion.

None of the measures employed caused a significant rise or fall in the glucuronide synthesizing power of the liver. Menthol causes a rise in the glucuronidase activity of the liver. Fishman (1940) explained this rise on the assumption that glucuronidase synthesizes glucuronides in vivo and that the rise in glucuronidase activity was an adaptation process caused by the administration of a glucuronidogenic substance. Levvy et al have shown that the rise in glucuronidase activity was a result of damage to the liver caused by menthol and subsequent regeneration process. This rise in glucuronidase activity occurred irrespective of whether the toxic substance administered was glucuronidogenic or not. If an adaptation process does take place in the liver, one would expect the glucuronide synthesizing system to increase

TABLE (16)

The effect of various measures on the glucuronide-synthesizing system in mouse liver

Treatment	Dose (g./kg.)	Days after treatment	o-Aminophenol conjugated/g dry weight (μg.)	Glucuronidase activity G.U./gm. wet weight
None	-	-	570 ± 43 (41)	273 ± 13 (23)
(-)-Menthol, intraperitoneally in olive oil	0.33	1	560 ± 40 (3)	823 ± 135 (3)
		3	640 (2)	953 ± 39 (3)
		6	650 (2)	775 ± 46 (7)
Carbon tetrachloride, subcutaneously in olive oil	5.33	1	560 ± 170 (3)	830 ± 62 (3)
		3	640 ± 200 (3)	763 ± 65 (3)
		7	640 ± 150 (3)	693 ± 31 (3)
Partial hepatectomy	-	3	670 ± 60 (6)	951 ± 115 (6)
		7	650 ± 50 (6)	1065 ± 62 (9)
		10	610 ± 80 (6)	657 ± 35 (3)
Oestrone, subcutaneously in olive oil (ovariectomized mice)	0.0017	4	580 ± 70 (3)	569 ± 73 (6)
Colchicine, subcutaneously in aqueous solution	0.0015	1	450 ± 80 (6)	273 ± 22 (3)
Sorbic acid, subcutaneously in aqueous solution	0.24	4	440 ± 70 (12)	27 ± 20 (12)
	0.16	4	540 ± 120 (6)	286 ± 37 (3)

(When the mean is based on values for individual animals the standard error of the mean for the group is also shown. Figures in brackets are numbers of animals used.)

in activity after administration of a glucuronidogenic substance.

Carbon tetrachloride is a liver poison. It causes an increased glucuronidase activity in the liver after its administration. It does not, however, come under the category of a glucuronidogenic compound.

Partial hepatectomy comes neither under the category of a liver poison nor a glucuronidogenic substance. This measure causes a stimulation of the growth process in the liver so that the liver cells are in a state of rapid proliferation.

Oestrone, could be both a stimulant of cell proliferation (cf. Kerr et al 1949) and a glucuronidogenic substance in view of the fact that it could be converted to oestriol in the animal body.

Colchicine in the dose shown causes no change in the normal glucuronidase activity of the liver, but it does prevent the rise in activity which follows such measures as partial hepatectomy. It acts as a mitotic poison. There is a slight suggestion of an inhibition of the synthetic activity.

Sorbic acid, in the smaller dose studied behaves like colchicine in its effect on the glucuronidase activity. With the higher dose there resulted a profound depression of the glucuronidase activity (Kerr et al 1950) both in liver and kidney. The slight suggestion of an inhibition of synthetic activity by sorbic acid and colchicine appear to be related to their actions as inhibitors of mitosis.

G) The effect of various compounds on the synthesis of o-aminophenylglucuronide by liver slices *in vitro*

Four of the compounds examined for their action on the glucuronide synthesizing system *in vivo* were tested *in vitro* for their effect on the conversion of o-aminophenol to its glucuronide by surviving liver slices from normal adult mice. Table (17) presents the results of these experiments. Oestrone was too sparingly soluble in water to permit its study in the present experiments.

TABLE (17)

The effect of various compounds on the synthesis of o-aminophenylglucuronide by mouse-liver slices

Compound	Concentration (M)	o-Aminophenol conjugated (µg./g. dry weight)		Inhibition (%)
		In controls	In presence of compound	
Sorbic acid	0.01	380	50	87
	0.01	970	370	62
	0.005	970	500	49
Colchicine	0.01	380	140	63
	0.01	530	190	64
	0.005	530	270	49
Carbon tetrachloride	0.0015	490	520	-6
	0.0015	660	520	21
(-)-Menthol	0.001	450	120	73

Colchicine, sorbic acid and menthol were added as aqueous solutions (if necessary, after pH adjustment) during the preparation of the bicarbonate Ringer solution. In the case of CCl_4, the Ringer solution was made saturated with the compound. In all experiments, controls were done with untreated slices from the same animal. Each determination was done in quadruplicate, and the standard error of the mean is thus about 10% (Levvy & Storey, 1949).

Sorbic acid does not inhibit β-glucuronidase *in vitro* (see previous section). Neither does colchicine. Both these substances appear to have an inhibitory effect on the glucuronide synthesizing system. Inhibition was almost 50% with 0.005 M colchicine or sorbic acid. Menthol in an even lower concentration causes 73% inhibition of the formation of o-aminophenylglucuronide. Whereas colchicine and sorbic acid cannot be classed as glucuronidogenic substances, menthol has been shown by Lipschitz & Bueding (1939) to form glucuronides in the presence of surviving liver slices. It is therefore very likely that the observed depressant action of menthol on the synthesis of o-aminophenylglucuronide *in vitro* is the result of competition with o-aminophenol to form a glucuronide rather than genuine inhibition of the synthetic mechanism.

The inhibitory actions of sorbic acid and colchicine on glucuronide synthesis *in vitro* are difficult to interpret at present. The overall synthetic process is known to be adversely affected by other agents such as cyanide, fluoride, iodo acetate (Lipschitz & Bueding 1939), azide and sulphate (Dr. I. D. E. Storey, private communication), but their mode of action is in most cases still not quite clear.

Sorbic acid, menthol, carbon tetrachloride and colchicine have no significant effect on the colour development of o-aminophenylglucuronide. Percentage recoveries of o-aminophenylglucuronide in the presence of these compounds are shown in Table (18).

TABLE (18)

The effect of various substances on the colour development of o-aminophenylglucuronide

Substance	Conc^{n} (M)	µg. o-aminophenylglucuronide estimated	% Recovery
None	-	126	-
Sorbic acid	.01	114	91
Carbon tetrachloride	.0015	121	96
Menthol	.001	123	98
Colchicine	.01	125	99

H) Attempted synthesis of o-aminophenylglucuronide by mouse liver β-glucuronidase preparations and liver homogenates

Florkin, et al (1942) claim to have demonstrated conjugation of borneol with glucuronic acid in presence of ox spleen glucuronidase. At the end of the incubation period, free glucuronic acid was removed with copper sulphate and calcium hydroxide and glucuronic acid in combination with borneol was estimated by the Tollens colour reaction. Only a very small fraction of the total glucuronic acid present was in the combined form even after incubation for several days.

The use of o-aminophenol as the aglucone in demonstrating glucuronide synthesis has the advantage that in the final reaction traces of the conjugated glucuronide give a pink colour which is never seen in controls.

Free o-aminophenol was incubated with glucuronic acid in the presence of concentrated purified preparations of mouse liver glucuronidase. No o-aminophenyl-glucuronide was detected at the end of the incubation.

D-glucurone was present in final concentrations varying from 0.4 to 0.125 M in 0.05 M citrate buffer at pH 5.2 or 0.05 M phosphate buffer at pH 7.4. The incubation mixtures contained 0.0025% o-aminophenol and 0.001 M ascorbic acid as in experiments with tissue slices. The mixtures were incubated with shaking at 37° C for periods of 2 and 22 hrs. In the longer term experiments, the incubation flasks were filled with N_2 to prevent oxidation of the free phenol.

In another series of experiments the liver slices in the procedure of Levvy & Storey for measuring the glucuronide synthesizing activity of the liver, were replaced by crude liver homogenates. In such preparations all the tissue constituents initially present are presumably still there except for the very labile molecules which may have been destroyed. The organised cell structure has also been upset. No synthesis of o-aminophenylglucuronide was observed in these experiments.

I) The effect of diet on the glucuronide synthesizing system of young mouse liver

The nature of the development of the synthesizing system in the liver of the mouse suggested that diet may have an effect on the rate of development of the synthesizing system. Young mice are usually weaned about the third week after birth when they start taking solid food. It was thought that if they were weaned earlier and kept on a solid diet, the glucuronide synthesizing system might develop faster.

Young mice from 4 litters, totalling 23 were separated from their mothers at 17 days of age and divided into two groups - the litters being split between the groups. One group was then kept with the mothers while the other was kept on a diet of rat cake. Group (1) thus had a choice of mothers' milk and solid food while group (2) had only solid food. At the end of a further 10 days when they were 27 days old, the livers of the mice from both groups were examined for their synthetic activity. The results were as follows:-

Group (1)	Mother's milk + solid food	490 ± 80 µg/g dry wt.
(2)	Solid food only	510 ± 60 µg/g dry wt.

The difference between the groups is not significant. The results suggest that diet did not influence the development of the synthesizing system in liver and that the development was dependant on the age alone. Attempts to wean the mice at an earlier age were unsuccessful as most of them died within a few days. If this could be done successfully, and the

synthetic activities of the liver measured at 3 weeks of age a significant difference between the two groups may well be obtained. It is felt that depriving the young mice of mother's milk - a possible source of glucuronides - before the synthesizing system is well developed would hasten the development of the synthesizing system on a solid diet.

Sodium sulphate

In another experiment 6 infant mice from the same litter 15 days old were separated into two groups. One group was fed ordinary rat cake while the other was fed rat cake containing 1% (w/w) Na_2SO_4. At the end of a further 4 weeks, the livers from the mice of both groups were examined for their synthetic activity. Those from the group fed on rat cake only had a synthetic activity of 770 units per gm. dried tissue while the group fed on rat cake containing Na_2SO_4 had an activity of 430 units. The number of animals used was small and therefore no definite pronouncement can be made on the point. There is the suggestion, however, of the Na_2SO_4 having suppressed the glucuronide forming system in the liver.

DISCUSSION

β-glucuronidase has been presumed to act synthetically *in vivo* by Fishman (1940). His evidence for this hypothesis was the fact that when menthol and borneol were administered to mice and dogs respectively the glucuronidase activity of liver, kidney and spleen in these animals was increased. The activity of the enzyme in ovary and uterus in the dog and ovary, uterus, testes and vagina in the mouse remained unaltered. In subsequent experiments (1944, 1947) he observed that the administration of oestrogens to ovariectomized mice caused an enhanced glucuronidase activity in uterus, but not in liver and other organs. It was necessary to postulate two physiologically different glucuronidases in the mouse although Fishman's own work gave no evidence for any differences between the enzymes from liver and uterus *in vitro*.

Levvy *et al* (1948, 1949) repeating Fishman's work have shown that the increased glucuronidase activity in the organs of the mouse was not caused solely by the administration of glucuronidogenic substances but by toxic substances in general as a result of damage in the organs concerned and subsequent cell proliferation. Increased glucuronidase activity has been associated with rapid cell proliferation in tissues. All Fishman's earlier results and the later results with cancer tissues in humans can be explained on Levvy's hypothesis (Fishman & Anlyan 1947; ~~1948;~~ McDonald & Odell 1947; Odell & McDonald 1948; Odell & Burt 1949, Odell & Fishman 1950). Moreover, the work of Levvy

et al suggests that there is no distinction in vivo between the uterine and liver enzymes in function, since, for example, oestrone and carbontetrachloride cause changes in both (Kerr et al 1950). In the light of all this evidence, the view that increased glucuronidase activity in a tissue is a result of the increased demand for glucuronide formation requires much stronger evidence to support it.

β-glucuronidase has been shown to be widely distributed in mammalian tissues (Oshima, Fishman, and Levvy et al). The glucuronide synthesizing system on the other hand has so far been found only in liver and to a smaller extent in kidney (Lipschitz & Bueding 1939). This would suggest that the two systems are distinctive. Evidence to prove that it is so, has been obtained in several ways.

A study of the glucuronide synthesizing system in the mouse has shown that the synthetic system is confined to liver and to some extent kidney. Spleen and lung which are rich in glucuronidase activity were devoid of synthetic power. So were the two transplantable tumours examined.

The synthesizing system in the liver develops with the age of the mouse. It is practically nil at birth and develops rapidly during the second and third weeks of its life to reach adult values about the fifth week. The β-glucuronidase activity on the other hand is high at birth, falls rapidly during the second and third weeks and reaches adult values about the fifth week. It should be noted that

a young mouse weans itself about 14-21 days after birth and starts to take solid food about the same time. It is therefore significant that the rapid development of the glucuronide synthesizing system and also the rapid fall in the glucuronidase activity in the liver should occur at this time.

That the observed synthetic power with liver slices from young mice is not the nett result of two opposing enzyme systems has been satisfactorily proved by the use of saccharic acid - an efficient inhibitor of glucuronidase activity in vitro. The results of experiments described in this section taken along with the findings of Campbell (1950) in his histochemical studies leave no doubt as to the penetration of saccharic acid into the cells, in the in vitro tissue slice experiments described.

Another line of approach has been the study of the glucuronide synthesizing system in liver under conditions which showed a marked change in the glucuronidase activity from normal. The injection of carbontetrachloride and menthol to normal mice or oestrone to ovariectomized mice or partial hepatectomy causes a rise in the glucuronidase activity of the liver (Levvy et al 1948, Kerr et al 1949, Fishman 1939). The glucuronide synthesizing power, however, showed no marked difference from normal under these conditions of increased glucuronidase activity.

Colchicine has no effect on the liver glucuronidase activity when administered in the dose described (Kerr et al 1950), but it arrests mitosis in the liver cells (Scheifley & Higgins 1940).

The glucuronide synthesizing power of the liver appears to be very slightly affected.

Sorbic acid has been shown by Kerr et al (1950) to reduce the glucuronidase activity in the liver of the mouse 4 days after injection of a suitable dose viz. 0.24 g/Kgm. Very often the activity was almost reduced to zero. The synthesizing power, however, did not show any marked changes from normal.

All these experiments have shown that while marked changes in the glucuronidase activity of the liver take place under suitable conditions, no parallel changes in the glucuronide synthesizing system are evident.

Yet another method of approach has been the study of the effect of saccharic acid on the in vitro synthesis experiments with tissue slices. Hydrolysis of a glucuronide by tissue slices is inhibited in the presence of saccharic acid. But when saccharic acid is added to the bicarbonate Ringer solution in the in vitro synthesis experiments only a very small depression in the synthetic activity has been noted. Furthermore this slight depressant action has also been shown to be caused by other dicarboxylic acids which have no action on glucuronidase activity in vitro.

While the inhibitory action of menthol under similar conditions is probably a competitive one, that of sorbic acid and colchicine is difficult to interpret at present.

Attempts to synthesize o-aminophenyl-

glucuronide by incubating either a purified preparation of β-glucuronidase or crude liver homogenates with excess glucurone and o-aminophenol in suitable buffers were unsuccessful. Florkin et al , however, have claimed slight success in this direction using ox spleen glucuronidase. Failure in such experiments may on the one hand be due to not selecting the right conditions for the reaction to take place, while on the other hand it may be due to not having the right enzyme system to catalyse the reaction.

All the evidence obtained in the experiments described in this section suggests very strongly that the glucuronide synthesizing system and the glucuronide hydrolysing system are two distinct enzyme systems.

S E C T I O N (iii)

SOME ISOLATED OBSERVATIONS IN CONNECTION WITH WORK ON β-GLUCURONIDASE

INTRODUCTION

This section deals with a few observations which were made during the course of the work and which have not been included in Sections (1) and (2).

A) Presence of β-glucosidase in mouse liver

Masamune (1934) in his studies on β-glucuronidase examined the effect of his enzyme preparations on various substrates. He found that it had no effect on β-glucosides except phenyl-β-d-glucoside. Graham (1946) in his study of the preparation and purification of the enzyme stated that his preparations of the enzyme did not hydrolyse phenyl-β-d-glucoside. The author found, however, that when the enzyme was prepared by the method of Kerr et al (1948), the supernatant from the centrifuged homogenate sometimes caused hydrolysis of phenyl-β-d-glucoside. This finding suggests the presence of a β-glucosidase in animal tissues. After precipitation of the glucuronidase from the supernatant by 50% saturation with ammonium sulphate, the glucuronidase preparation lost the ability to hydrolyse phenyl-β-d-glucoside. The experiments of Pryde & Williams (1936) in which phenyl-β-d-glucoside fed to or injected into rabbits resulted in the excretion of ethereal sulphate, show that the β-glucoside was hydrolysed in the animal body to give free phenol. How the hydrolysis was accomplished was, however, not known.

The ability of glucuronidase preparations from mouse liver to hydrolyse β-glucosides would thus

appear to depend on the purity of the enzyme preparation.

B) A "yeast" like organism possessing β-glucuronidase activity

After a bottle containing phenyl-β-d-glucuronide solution had been accidentally left unstoppered overnight, it was found to contain a "yeast" like organism. Free phenol was found in the glucuronide solution. The organism was isolated by Dr. Wright of the Bacteriology Department, University of Edinburgh, and was found to liberate free phenol from phenylglucuronide solutions. Unfortunately, however, on further subculture in a synthetic medium it lost its glucuronidase activity. Attempts to restore the activity by introducing glucuronides into the synthetic medium were unsuccessful. Strains of E. Coli (Buehler et al 1949), Staph. Aureus (Barber et al 1948) have been shown to develop glucuronidase activity when grown on suitable media. The enzyme in these two organisms appear to be "adaptive" and not "constitutive" as a few strains of E. Coli and Staph. Aureus when tested for glucuronidase activity showed none.

C) Attempted synthesis of phenyl-β-d-glucuronide

Most of the β-glucuronides have so far only been prepared biosynthetically by feeding animals the "aglucones" and isolating the conjugated glucuronides, excreted in the urine, by suitable methods (See publications from the laboratory of R. T. Williams, Liverpool & London). The selective oxidation of sugars is an old problem in

carbohydrate chemistry. The oxidation of a reducing group to a carboxylic group or both end groups in a sugar to carboxylic groups has been quite easily achieved. The direct terminal oxidation of a sugar whilst retaining an aldehyde group, however, still presents great difficulties. Reichstein (1938) and the Americans (1939) have achieved a certain amount of success in preparing glucuronic acid synthetically by partial reduction of saccharic acid in the first instance and by anodic oxidation of alkylglycosides in the second. The yields, however, were poor in both cases.

Maurer & Drefahl (1942) have succeeded in preparing methylgalacturonic and methylglucuronic acids by oxidising the corresponding galactoside and glucoside with N_2O_4 in an inert solvent - viz. chloroform. Dr. Kerr in this laboratory attempted the oxidation of phenyl-β-d-glucoside under similar conditions. She has reported success only in one instance (Ph.D. Thesis 1949). In her later experiments she could not isolate the phenyl-β-d-glucuronide, if any had been formed.

The author has attempted several times to oxidize phenyl-β-d-glucoside to phenyl-β-d-glucuronide using N_2O_4 in chloroform, but invariably ended up with a brownish coloured solid which showed acidic properties and turned a dark brown in alkaline solutions. There was a strong suggestion that it may have been a nitrated phenyl-β-d-glucuronide.

This problem was abandoned for the time

being, but the author hopes to return to it at some later date.

D) Modification of the phenolphthalein glucuronide method of Talalay et al for the assay of β-glucuronidase activity

Talalay et al (1946) developed a method using phenolphthalein glucuronide as substrate for the assay of β-glucuronidase. The estimation was carried out as follows:- 4.0 ml. of 0.1 M acetate buffer, 0.5 ml. of 0.1 M phenolphthalein monoglucuronide solution and 0.5 ml. of an enzyme preparation were incubated together for an hour. At the end of the incubation, 5.0 ml. of 0.4 M glycine buffer pH 10.45 was added to the incubation mixture to stop the reaction and at the same time develop the colour of the phenolphthalein liberated.

One of the disadvantages of this method was that protein was not completely precipitated by the addition of glycine. Fishman et al (1948) found that it was necessary to modify this method as the incomplete precipitation of protein was a source of error in the estimation of human blood glucuronidase activity. Consequently, they stopped the enzyme action by adding trichloroacetic acid which also precipitated the protein. The tubes were then centrifuged and the supernatant poured out into another tube. The sediment was resuspended in a small quantity of water and centrifuged again. The supernatant from this was added to the first, the volume of the supernatant and washings made up to a known volume and the colour now developed by the addition of a mixed NaOH - glycine buffer.

When this modified method was used by the author in preliminary studies using enzyme preparations containing large amounts of inactive cell debris, very variable results were obtained. It was therefore decided to carry out recovery experiments when phenolphthalein was added to enzyme preparations. While almost 100% recoveries were obtained with the original method of Talalay *et al*. when using ammonium sulphate precipitated enzyme fractions, the recoveries with enzyme preparations containing much inactive cell debris ranged from 84 - 95% depending on the amount of cell debris present. In the modified method of Fishman while 71% recovery was obtained with a debris free supernatant, 62% was obtained when cell debris was present - see table (19). The precipitate thrown down by the trichloroacetic acid would thus appear to adsorb a large percentage of the added phenolphthalein.

With mouse liver preparations, it was found that the homogenate from 1 g. liver could be diluted to 40 ml. in water to give fairly reasonable enzyme activity and at the same time to contain small enough quantities of protein and cell debris to give almost 100% recoveries of added phenolphthalein. Under these conditions, the original method of Talalay *et al* (1946) was quite satisfactory.

TABLE (19)

Percentage recoveries of added phenolphthalein

Method	Nature of enzyme preparation	µg phenolphthalein		%
		Added	Recovered	Recovery
Talalay et al (1946)	Supernatant from unincubated homogenate	24.3	24.0	99
		49.0	48.4	99
	Supernatant from incubated homogenate	24.3	23.4	96
		49.8	49.7	100
	Debris from unincubated homogenate	24.3	20.6	84
		49.0	44.8	91
	Debris from incubated homogenate	24.3	23.1	95
		49.8	43.5	87
Fishman et al (1948)	Supernatant from incubated homogenate	21.3	15.0	71
	Debris from incubated homogenate	21.3	13.0	61

If the initial hydrolysis was carried out at varying pH, the addition of glycine buffer did not always bring the final pH of the solution to the range where maximum development of the phenolphthalein colour was obtained. This was recognized by Talalay et al in their pH optimum experiment and a suitable modification was employed when

they studied the pH optimum of their enzyme preparations.

The author has found that the addition of 0.25 M Na_2CO_3 to the glycine buffer, partially solves this difficulty - though not entirely. Using a glycine - Na_2CO_3 mixed buffer, it has been found possible to compare the enzyme activities over the pH range 4.0 - 7.0 quite easily without having to go through the tedious titration procedure of Talalay et al. See table (20).

TABLE (20)

Comparison of glycine buffer (0.4 M) and glycine (0.4 M) - Na_2CO_3 (0.25 M) mixed buffer on the colour development of phenolphthalein at different pHs in 0.1 M acetate buffer.

(Figures denote Spekker readings)

pH	Glycine	Glycine - Na_2CO_3
7.0	198	198
5.2	188	202
4.5	171	196
3.4	059	178

Subsequent experiments using this mixed buffer have also shown that recoveries of added phenolphthalein are much better even in the presence of considerable quantities of inactive cell debris, than when glycine was used alone.

The modified procedure now adopted

is as follows:-

A mixture of 3.0 ml 0.1 M buffer
0.5 ml 0.1 M substrate
0.5 ml enzyme preparation

is incubated for an hour at 37°C. At the end of the incubation 4.0 ml of 0.4 M glycine buffer containing 0.25 M Na_2CO_3 is added to stop the reaction. The tubes are centrifuged at 3000 r.p.m. for 10 mins. on a bench centrifuge and the colour of the clear supernatant solution measured on a Spekker absorptiometer using yellow green filter Ilford No. 605.

E) Heparin and ascorbic acid as inhibitors of β-glucuronidase

Becker & Friedenwald (1949) reported inhibition of rat tissue glucuronidase by heparin and ascorbic acid. In their estimations, a substrate concentration of 0.0005 M was employed although in the original Talalay et al procedure the concentration of the substrate was 0.01 M. When the experiments of Becker & Friedenwald were repeated with mouse liver glucuronidase preparations and using the original Talalay et al procedure, only moderate inhibitions were observed. The results of these experiments are expressed in Table (21).

TABLE (21)

Effect of heparin and ascorbic acid on mouse liver glucuronidase preparations

Compound	Conc^{n}	Enzyme preparation	μg phenolphthalein liberated		% Inhibition	* % Inhibition with rat liver prep^{s}
			In controls	In presence of inhibitors		
Heparin	40 mg%	Crude homogenate	28.6	18.8	34	85
		$(NH_4)_2SO_4$ ppt^{d} fraction	22.2	10.6	52	52
Ascorbic acid	20 mg%	Crude homogenate	28.6	23.0	20	-
		$(NH_4)_2SO_4$ ppt^{d} fraction	22.2	11.0	50	83

* Figures in this column quoted from Becker & Friedenwald (1949).

The higher percentage inhibition obtained by Becker & Friedenwald in some instances may have been either due to species difference or the lower substrate concentration employed in their estimation. If it is necessary to dilute the substrate to obtain inhibition, the substances must be very feeble inhibitors; hence the failure to find it in our own study of inhibitors.

F) Glucuronide synthesizing system in chicken liver

Chicken liver was examined for its ability to synthesize O-aminophenylglucuronide under the same conditions employed for mouse liver slices. Although it had a glucuronidase activity of 228 G.U. per g. moist tissue (Kerr Ph.D. Thesis 1949), it had no synthetic activity. The failure to observe synthetic activity may have been due to the unfavourable conditions employed for the study of glucuronide synthesis with chicken liver slices. The author has found references in the literature in which glucuronic acid is claimed to be a growth factor for chickens (Robinson et al 1939, Hegsted et al 1939, Almquist et al 1940 (a), 1940 (b).). It would be quite interesting to study both the glucuronidase activity and the glucuronide synthesizing system in the different tissues of the chicken at various ages. In contrast to unweaned young mice, it should be possible to keep chickens on a strictly controlled synthetic diet from the date of hatching.

GENERAL DISCUSSION

GENERAL DISCUSSION

The function of β-glucuronidase *in vivo* still remains one for speculation. The results of the present work firmly establish the fact that the glucuronide synthesizing system and the glucuronide hydrolysing system are quite distinct. While the former appears to be a complex system, confined mainly to liver and to some extent the kidney in the organs examined so far in the mouse, the latter, β-glucuronidase, has been found in practically all organs examined. This observation by itself should be enough evidence for maintaining that the two systems are different. Measures causing a rise or fall in glucuronidase activity in mouse liver have not caused a corresponding rise or fall in the synthetic activity of the liver showing that there was no direct relationship between the two enzyme systems. Finally, some organs having high glucuronidase activity (young mouse liver and cancer tissues) showed very small or no synthetic activity at all. These findings effectively dispose of Fishman's postulate of a synthetic role for β-glucuronidase in the animal body.

Is glucuronide formation merely a detoxication mechanism? The answer is - 'No'. Although it has been regarded purely as a detoxication process for quite a long time, the isolation of several physiologically important substances as their glucuronides and also the widespread presence of β-glucuronidase in the animal tissues

have now made workers regard glucuronide formation as one of the metabolic conjugation processes.

One may regard the glucuronide synthesizing system and β-glucuronidase as forming a group of enzymes regulating the transport of certain substances to the various tissues in the animal body. The required substance may either be the glucuronic acid molecule or the aglucone molecule or both. Muco-proteins containing glucuronic acid residues enter into the composition of certain types of tissue. It is therefore possible that when glucuronides are transported in the blood stream to these tissues, β-glucuronidase acts on them liberating free glucuronic acid for the formation of mucoproteins. Another substance that may possibly be built up thus is hyaluronic acid. The glucuronides of the steroid hormones on the other hand may be hydrolysed mainly for the sake of the steroid action in organs such as the uterus.

Salkowski & Neuberg have reported the presence of glucuronic acid decarboxylase in bacteria isolated from putrefying flesh. The decarboxylation of glucuronic acid would give xylose. But one cannot regard it as impossible for the animal body to convert xylose to ribose and then use it in the synthesis of nucleic acids.

In a rapidly proliferating tissue, the glucuronidase activity is increased. If the function of glucuronidase is to liberate glucuronic acid from glucuronides for the formation of new cell constituents where do

the glucuronides come from? Either it is synthesized in some other organ in the animal or is taken in with ingested food or as in a damaged organ it may come from the damaged tissue itself. In an adult mouse which has been administered a toxic compound such as menthol or chloroform, the synthetic activity of the liver is not increased. In such cases one might expect endogenous glucuronides ~~to~~ from damaged cells to supply the necessary glucuronic acid for the formation of new cell material. In a young mouse, the synthetic system in the liver does not develop till about the third week. Up to the third week or so, the mouse lives mainly on its mother's milk and starts taking solid food only after the third week. It is highly probable that as the organs in a young mouse show high glucuronidase activity and are all in a state of rapid growth, glucuronides are provided to the young animal in the mother's milk. The author has on various occasions considered the possibility of testing this hypothesis, but has for various reasons postponed the investigation.

It is unfortunate that the inhibitor discovered for β-glucuronidase does not produce any in vivo effects. A substituted saccharic acid which is not easily metabolised in the animal body should be of great help in the elucidation of the physiological role of β-glucuronidase.

The discovery of a glucuronic acid decarboxylase in animal tissues would not be very surprising.

Certain bacterial polysaccharides are

known to contain glucuronic acid residues. Studies with these bacteria might yield some valuable information regarding glucuronic acid metabolism.

Glucuronic acid is claimed to be a growth factor for chickens. If, as was observed in the single experiment, chicken liver has no glucuronide synthesizing activity, but has glucuronidase activity, it should prove to be suitable material for investigation of the possible function of β-glucuronidase *in vivo*.

It might be interesting to speculate why a different enzyme system should be necessary to promote synthesis of glucuronides. The work of Lipschitz & Bueding suggests very strongly that a phosphorylating mechanism is involved during the synthesis and also that the oxidative activity of the cell is an essential requirement. Substances like adenosine triphosphate (A.T.P) and creatine phosphate (C.P.) have been shown to take part in energy transfers via energy rich phosphate bonds during cell metabolism. If glucuronide formation is a process involving large energy transfers, then a mechanism involving a substance such as A.T.P. or C.P. would be necessary for its accomplishment.

It seems possible that the synthetic system in liver forms glucuronides from 3-carbon compounds by such reactions and that glucuronidase in liver and other tissues liberates from the glucuronides formed free glucuronic acid to meet their requirements.

SUMMARY

SUMMARY

1). The inhibitory effect of some substances on β-glucuronidase activity has been investigated. D-gluco saccharic acid is the most powerful of the inhibitors that have been studied. 50% inhibition was obtained with 2×10^{-4} M saccharic acid concentration while almost 100% inhibition was obtained with 10^{-2} M saccharic acid.

2). The inhibitory effect of saccharic acid appears to be independant of the source of the enzyme and the degree of purity of the preparation. Liver, uterus, kidney, spleen, and tumour enzyme preparations are all inhibited equally well. The degree of inhibition does not appear to be markedly affected by the concentration of the enzyme preparation.

3). The inhibition appears to be of a reversible nature. Precipitation of the enzyme from a solution containing saccharic acid by 50% ammonium sulphate saturation gives a precipitate which is almost free of any loosely combined saccharic acid. A second precipitation with ammonium sulphate frees the enzyme of the saccharic acid completely.

4). An approximate value for K_I - the dissociation constant of the enzyme inhibitor complex has been obtained for the β-glucuronidase saccharic acid complex.

5). No in vivo effects were produced when saccharic acid was administered to mice either orally or by injection. This is possibly due to the rapid destruction of saccharic acid in the animal body.

6). The glucuronide synthesizing system has been studied in some tissues of the mouse. The activity is mainly confined to liver and to a smaller extent in the kidney. β-Glucuronidase activity on the other hand has been shown by other workers to be widely distributed in the animal body.

7). The glucuronide synthesizing system in the mouse liver develops with the age of the mouse. It is practically nil at birth and approaches steady adult values about the fifth week after birth.

8). The β-glucuronidase activity of a tissue does not affect its glucuronide synthesizing activity. This has been demonstrated by the use of saccharic acid in tissue slice experiments.

9). Saccharic acid has been shown to penetrate tissue slices. Our results have been confirmed by the findings of Dr. J.G. Campbell, using a different technique.

10). Saccharic acid has no marked effect either on the aerobic or anaerobic respiration of mouse liver slices when tested in the Warburg apparatus.

11). Measures, such as partial hepatectomy or injection of menthol, carbon tetrachloride, or sorbic acid, which cause a rise or fall in the glucuronidase activity of mouse liver, did not show corresponding changes in the glucuronide synthesizing power of the liver.

12). Sorbic acid, colchicine, and menthol, have been shown to have an inhibitory effect on the <u>in vitro</u> synthesis of glucuronides by mouse liver slices. While the effect of menthol may be purely competitive, that of sorbic acid or colchicine is difficult to explain at the moment.

13). Attempts to synthesize o-aminophenyl glucuronide by incubating o-aminophenol, glucuronic acid and β-glucuronidase preparations or tissue homogenates have proved unsuccessful.

14). Preliminary studies on the effect of diet on the glucuronide synthesizing system of mouse liver have been reported.

15). The presence of a β-glucosidase in mouse liver preparations has been observed.

16). A "yeast like" organism possessing β-glucuronidase activity was isolated from a solution of phenylglucuronide, but was found to lose its activity on further subculture in a synthetic medium.

17). An unsuccessful attempt at synthesizing phenyl-β-d-glucuronide by the oxidation of the glucoside with N_2O_4 in chloroform has been reported.

18). A modification of the Talalay et al procedure for the assay of β-glucuronidase activity using phenolphthalein glucuronide has been described.

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PART (2)

A GLUCURONIDE-DECOMPOSING ENZYME IN THE RUMEN OF THE SHEEP

INTRODUCTION

At one end of the scale in the animal species there are the carnivores with a comparatively simple alimentary tract. At the other end come the herbivores with a much more elaborate digestive apparatus. The omnivores occupy an intermediate position in this respect.

The food ingested by an animal may be broken down, generally speaking, by three different mechanisms; - by enzymes secreted into the alimentary tract by the animal, by enzymes taken in along with ingested food or by micro-organisms occurring in the alimentary tract of the animal. In carnivores and man, as far as is known, the food ingested is broken down by enzymes secreted by the animal itself into the alimentary tract. In the herbivores however, the position is different. The greater portion of their diet is composed of cellulose and other complex polyuronides in which the individual sugar units are linked to one another by β-linkages. Although enzymes capable of breaking down polysaccharides containing α-glucosidic linkages have been found in the digestive juices of these mammals, no enzyme capable of breaking down β-linked polysaccharides has as yet been identified. It is generally agreed that the breakdown of such complex β-linked polysaccharides is brought about by the micro-organisms present in the alimentary tract.

The alimentary tract of the herbivores differs from that of the carnivores chiefly in respect of

capacity. This is the basic adaptation to the herbivorous mode of life. The most usual form is the enlargement of the caecum or colon as is well marked in the equidae. The ruminants on the other hand, in addition to an enlarged caecum and colon, possess a modified and capacious gastric system. The ruminant stomach is in four parts - the rumen, reticulum, omasum and abomasum. The first two, rumen and reticulum, together function as a single unit. The solid food enters the rumen while saliva and water enter the reticulum. Owing to the brisk regular contractions of the reticulum which occur about once a minute, the contents of the reticulum are washed into the rumen there to be mixed thoroughly with the food particles. Later, the contractions of the walls of the rumen return the fluid to the reticulum. By this process, a gradual separation and accumulation of the smaller food particles occurs in the reticulum from where they pass on through the omasum into the abomasum where true gastric digestion takes place. Of the four compartments described above, only the abomasum is glandular.

The rumen is well suited to the maintenance of a large and active population of micro-organisms. The contents of the rumen are kept continually stirred by the muscular contractions of the walls of the rumen. A copious and continuous supply of saliva not only serves as a medium for the suspension of food particles but also acts as a good buffer for the growth of the rumen micro-organisms.

It has been well established as a

result of the work of Tappeiner (1884), Thomas & Pringsheim (1918) and others that cellulose ingested by a ruminant is broken down in the rumen of the animal. That this breakdown was caused not by an enzyme secreted by the animal but by micro-organisms has also been established by Tappeiner (1884) and Scheunert (1906). Gray (1947) has shown that in the sheep the digestion of cellulose occurring in the rumen could be as much as 70% of the ingested cellulose. The conditions obtaining in the rumen are anaerobic and hence the organisms causing the breakdown of cellulose and other polysaccharides have been regarded as mesophilic anaerobes.

During the breakdown of ingested food in the rumen of the sheep, carbondioxide and methane are liberated in large quantities, the micro-organisms increase in numbers and volatile fatty acids such as acetic, propionic and butyric are produced. Bacteria in common with other living things require both energy and nitrogen apart from growth factors and minerals. Furthermore, for effective digestion of insoluble material such as cellulose, bacterial growth is essential. Therefore it is reasonable to assume that the rumen micro-organisms live upon the food eaten by the animal and that any foodstuff entering the rumen is liable to attack by the micro-organisms.

Normally the amount of mono- and di-saccharides contained in the ruminant food is small. When glucose, fructose, cane sugar and maltose were introduced into the rumen, they were found to disappear rapidly, while

rapid fermentation occurred in the rumen resulting in the production of volatile fatty acids. Young pasture grass contains a large amount of fructosan - a water soluble polysaccharide - and other water soluble sugars in smaller amounts. The speed with which glucose disappears in the rumen, makes it unlikely that these soluble sugars would escape fermentation in the rumen. It has been shown by Kellner (1900) that starch and pure finely divided cellulose had the same nutritive value in the ruminant.

After a meal, certain types of cocci present in the rumen of the sheep were found to accumulate polysaccharides within their cell structure which later disappeared probably as a result of bacterial metabolism. This appearance and disappearance of a starch-like polysaccharide has been followed by their staining reaction with iodine. The organisms are known as "iodophiles" (Baker et al 1947-1948). This phenomenon is very marked when starch or glucose is fed to the animal.

Cultures of rumen micro-organisms when grown in vitro under suitable conditions on a cellulose medium have been shown to accumulate glucose, lactic and pyruvic acids in the medium. (Pringsheim 1912, Woodman & Stewart 1928, Woodman 1930, Woodman & Evans 1938.). The production of free glucose in the rumen, however, has never been demonstrated. Although some workers seem to think that part of the glucose may be absorbed as such from the rumen, the majority of workers in this field believe that any absorption from the rumen is by

way of volatile fatty acids formed from glucose via lactic or pyruvic acids (Marshall & Phillipson 1945, Phillipson 1942, Elsden et al 1945-1946, Phillipson & McAnally 1942, Barcroft et al 1944, Danielli et al 1945). One may therefore picture the complex food entering the rumen as being broken down by the micro-organisms to simpler units and part of it built up into their own cell structure (Baker 1939). Later this cellular material may be broken down either in the rumen itself during bacterial metabolism to produce volatile fatty acids or in the abomasum under conditions unfavourable for the existence of micro-organisms. That micro-organisms are easily disintegrated in the abomasum has been well established.

Several workers have tried to identify the organisms responsible for the breakdown of cellulose and other high molecular weight polysaccharides. Some have attempted to isolate the organisms in pure cultures on cellulose containing media (Pochon 1935, 1938). A certain amount of success in this direction has been achieved by these workers. Others have attempted a microscopic study of rumen contents and have found that certain types of cocci are found very closely attached to particles of food. They appear to be in cavities formed by the enzymic breakdown of the food particles. (Baker 1933, Baker & Martin 1937 a, b; 1939). These organisms are iodophilic and are believed to contain the cellulose decomposing enzyme. While in the former method there is the danger that a normally poorly growing organism in the rumen may grow vigorously on a synthetic medium and thereby gain

undue recognition as a potential source of cellulose digesting enzyme, the latter method is not quite complete by itself in identifying the organisms responsible for the breakdown of cellulose and other polysaccharides. Before an organism can be said to be a functional member of the rumen population, it should be shown to carry out a chemical reaction known to occur in the rumen and it should also be present in sufficient numbers in the rumen to carry out this reaction.

The micro-organisms in the rumen have been shown to change both qualitatively and quantitatively with the nature of the diet. These changes have been correlated to some extent with changes in the chemical activity of the microbial population (Elsden 1945, McDougall 1945, Quin 1943). Iodophilic cocci (Van der Wath 1942) "Yeast-like" organisms also called Quin's organisms (Quin 1943), a Gram negative iodophilic coccus and a Gram negative rod like organism (Hungate 1947, Sijpesteijn 1948) have been claimed to be associated with cellulose breakdown in the rumen of various animals. Strains of propionic acid bacteria have also been obtained (Elsden 1945). Some strains of Diplodinia and Entodinium of the protozoa present in the rumen are claimed by Hungate to possess cellulase activity (1942, 1943). Whether protozoa play as important a part as the bacteria in the nutrition of ruminants is a subject of much controversy.

Although quite a lot of attention has been paid by workers in the field of ruminant nutrition to the nature and absorption of the end products of digestion in the

rumen, very little work, if any, has been done on the initial stages of the breakdown of complex polysaccharides. As far as the author is aware, no study of an enzyme isolated from the rumen organisms has been made. Hungate (1942, 1943) has stated that cell free preparations from some strains of Diplodinia grown on a synthetic medium containing ~~glucose~~ cellulose had cellulase activity. Fahraeus (1947) has prepared a cell free preparation of an enzyme from Cytophaga globulosa - isolated from beech wood litter in the vicinity of Berlin - which was able to hydrolyse cellulose, lichenin and cellophane. The enzyme was extracted from a dried preparation of the organism with borate buffer at pH 9.0.

The present work was started with a view to a study of the hydrolytic enzymes involved in the initial breakdown of the carbohydrates normally present in ruminant foodstuffs. Instead of trying to isolate pure cultures of organisms growing in media containing specific polysaccharides found in ruminant foodstuffs, it was decided to make cell free enzyme extracts from a mixed rumen microbial population and after purification of such preparations to characterize the enzymes.

The technical difficulties associated with the preparation of cell free extracts of bacterial enzymes can now be overcome as a result of the work of Gale and others. The use of such extracts avoids the complication introduced by the rapid further metabolism of the initial breakdown products by the micro-organisms.

The detection of traces of hexoses and other initial hydrolysis products of a polysaccharide in a complex medium is very difficult, particularly when the initial hydrolysis is kept at a low rate by the insolubility of the substrates. The use of a simple soluble molecule containing the characteristic linkage - found in the complex polysaccharides present in ruminant foodstuffs - and giving rise to easily detected products, stable in the medium employed was considered hopeful for initial work on the enzymes. Once obtained in a comparatively pure and concentrated form, the study of the action of the enzymes on the natural substrates is simplified.

A β-glucuronide decomposing enzyme has been found in the micro-organisms present in the rumen of the sheep and some of its properties have been studied. A fuller investigation of the enzyme is in progress and the author hopes to publish the results of these investigations at a future date.

EXPERIMENTAL

A) ESTIMATION OF ENZYME ACTIVITY

Preparation of cinchonidine salt of phenolphthalein monoglucuronide

The cinchonidine salt of phenolphthalein monoglucuronide was prepared by the method of Fishman, Springer & Brunetti (1948). Rabbits were injected subcutaneously with a neutral solution of phenolphthalein phosphate daily for six days. The urine was collected each day, strained through surgical gauze and acidified with hydrochloric acid to Congo red paper. The acidified urine was extracted with ethyl acetate and the cloudy ethyl acetate layer centrifuged. The clear ethyl acetate supernatant was siphoned off and decanted through cotton wool. The ethyl acetate extracts from the six day's collection of urine were pooled, concentrated in vacuo to a small volume and the phenolphthalein glucuronide precipitated as its cinchonidine derivative. The crude product was recrystallized from a mixture of methylalcohol and ethyl acetate. The recrystallized product on hydrolysis with dilute hydrochloric acid gave 38.1% phenolphthalein, corresponding to the monoglucuronide.

Preparation of 0.1 M phenolphthalein glucuronide

0.788 g cinchonidine derivative of phenolphthalein glucuronide, 2.0 ml 6 N HCl, 30 ml distilled water and 20 ml ethyl acetate were mixed together with

stirring till the solid dissolved. The contents were transferred to a separating funnel and the ethyl acetate layer separated. The aqueous layer was extracted four times with 10 ml portions of ethyl acetate and the ethyl acetate extracts were all pooled. The ethyl acetate extract was decanted through a cotton wool plug and then evaporated to dryness in vacuo. The solid residue was taken up in water, the pH of the solution adjusted to 6.1 (glass electrode) and the volume made up to 100 ml.

Glycine - Sodium carbonate buffer

16.30 g aminoacetic acid and 12.65 g sodium chloride were dissolved in water and 10.9 ml of a concentrated sodium hydroxide solution (100 g NaOH in 100 ml water) added. The pH of the solution was adjusted to 10.45 and then 26.5 g anhydrous sodium carbonate added. The solution was finally made up to 1000 ml. The final pH was 10.7.

Procedure for the assay of the glucuronide-decomposing enzyme

3.0 ml phosphate buffer(0.1 M, pH 6.1,) 0.5 ml 0.1 M phenolphthalein glucuronide and 0.5 ml enzyme preparation were incubated together for an hour. At the end of the incubation, 4.0 ml of glycine - sodium carbonate buffer were added. The incubation mixtures were centrifuged for 10 mins. at 2700 r.p.m. on a bench centrifuge and the pink colour of the clear supernatant measured on a Spekker absorptiometer using Ilford filter No. 605 (Spectrum - yellow green).

Enzyme activities are usually expressed as μg phenolphthalein liberated per hour at 37°C. The amount of phenolphthalein liberated was read off a standard curve.

B) NITROGEN DETERMINATIONS

Catalyst for digestion (Chibnall, Rees & Williams 1947)

The catalyst was composed of a mixture of the following substances in a finely powdered form.

Potassium Sulphate	$K_2 SO_4$	80 g
Copper Sulphate	$Cu SO_4 . 5H_2O$	20 g
Sodium Selenate	$Na_2 SeO_4 10H_2O$	0.34 g

Mixed indicator

A solution of 33 mg. Bromo cresol green and 66 mg. Methyl Red in 100 ml of ethanol.

Boric acid reagent

40 g Boric acid were dissolved in 1520 ml ethanol and 80 ml of the mixed indicator added. The solution was made up to 2000 ml with distilled water.

Procedure

A suitable aliquot of the enzyme preparation was digested with 2 ml Conc. H_2SO_4 and a little of the catalyst for 8 hrs. The digest was transferred to the Markham steam distillation apparatus (1942), and after the addition of caustic soda, the ammonia liberated steam

distilled into a known volume of Boric acid reagent. When 25 ml of distillate had been collected, the distillate was titrated against $\frac{N}{100}$ HCl solution from a microburette till the green colour of the indicator turned pink. Suitable blanks were carried out with the reagents used.

1 ml N/100 HCl = 144 g N.

RESULTS

A) Description of sheep used and method of sampling rumen contents

A castrated male Cheviot sheep with a permanent rumen fistula was used in most of the experiments. The sheep was kept on a diet of hay and concentrates composed of 2 parts by weight of linseed oil cake and 1 part by weight of crushed oats. 150 g hay was fed twice a day at 8 a.m. and 8 p.m. and 150 g concentrates at 11 a.m. Rumen samples were taken $2\frac{1}{2}$ hrs. after the morning feed of hay using a wide bore glass tube. The volume of sample taken was usually about 500 ml and the samples were regarded as fairly representative of the rumen contents.

B) Microscopic examination of sample

The rumen sample as taken by the procedure described above contained large particles of hay and other foodstuffs in addition to the micro-organisms and

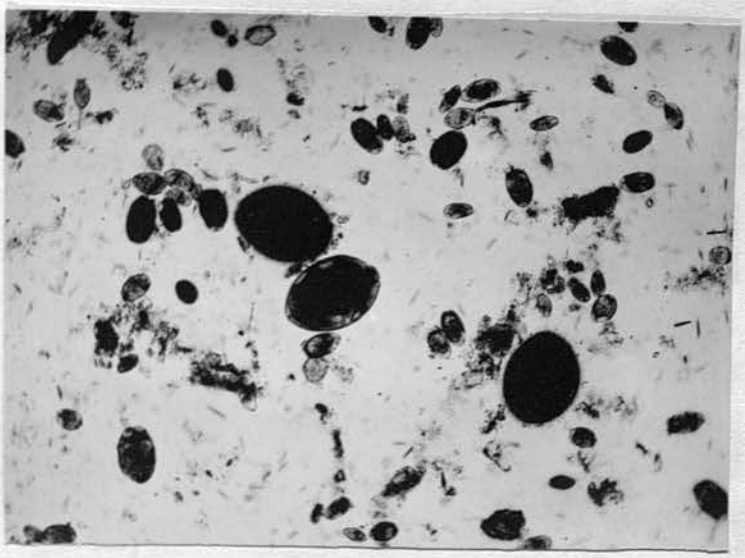
smaller food particles. The rumen liquor was always strained through eight layers of surgical gauze to remove the large particles of hay. A microscopic examination of the strained rumen liquor revealed a variety of protozoa, "yeast like" organisms - often referred to as Quin's organisms - "cigar shaped" bacteria and cocci. Small particles of hay were also found. Although no count of the organisms was made at any time, protozoa and Quin's organisms appeared to predominate.

c) Separation of the micro-organisms into fractions by fractional centrifugation

As the organisms present in the rumen were of different sizes, it was found possible to separate them into groups based on their relative sizes. Groups of micro-organisms obtained by various fractionation procedures were studied for their glucuronide-decomposing enzyme activity. As a result of this preliminary investigation the procedure finally adopted for the fractionation of the rumen micro-organisms was as follows:-

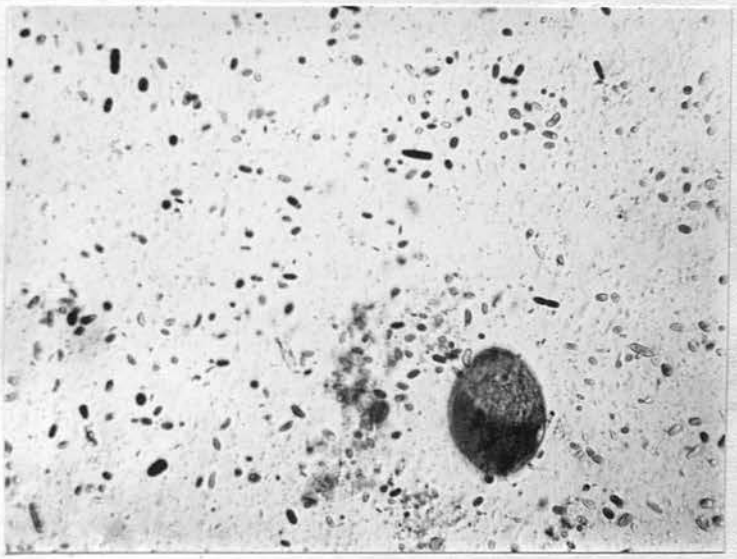
The strained rumen liquor was centrifuged for 2 mins. at a speed equivalent to an R.C.F. value of 14 x g. The greenish white sediment obtained contained plant debris, most of the protozoa and some of the large sized bacteria and Quin's organisms. See fig. (1). The supernatant was now centrifuged for 30 mins. at a speed corresponding to an R.C.F. value of 1540 x g. The creamy white sediment obtained consisted mainly of Quin's organisms, the larger

Fig. 1.



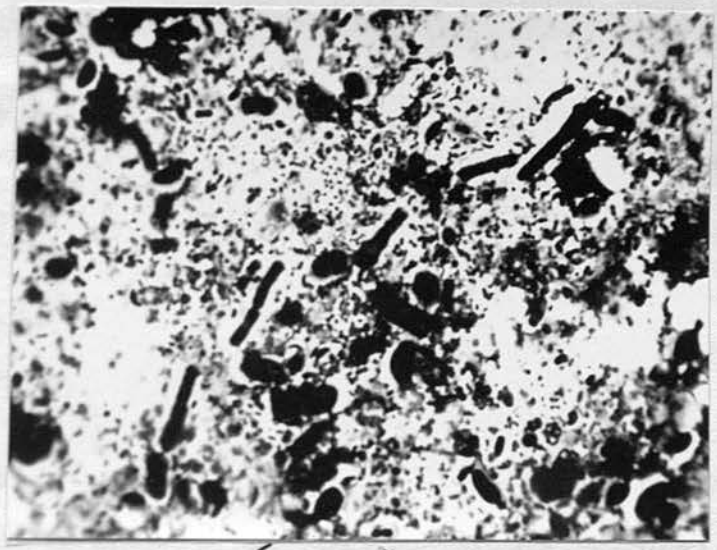
(x 100)

Fig. 2.



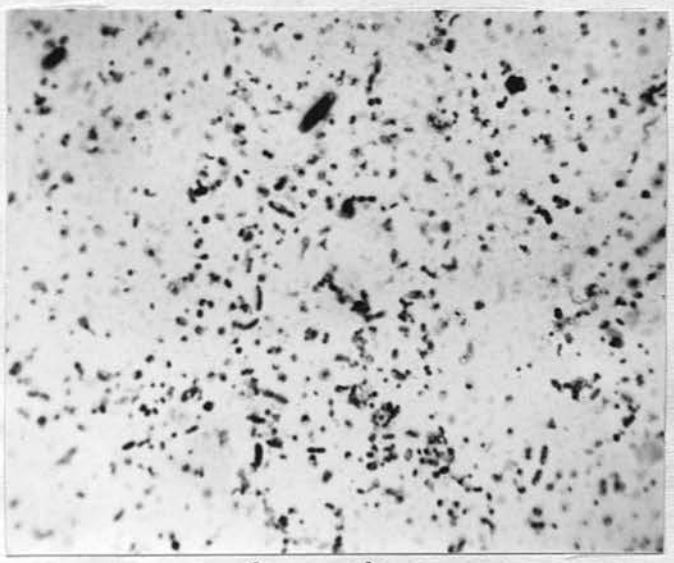
(x 390)

Fig. 3.



(x 1000)

Fig. 4.



(x 1000)

bacteria and some protozoa. See figs. (2) and (3). The supernatant was next centrifuged for 30 mins. at a speed corresponding to an R.C.F. value of 5140 x g. The white sediment obtained consisted of the smaller bacteria and a few large bacteria and Quin's organisms. See fig. (4). The supernatant from this centrifugation was a dark brown and almost cell free.

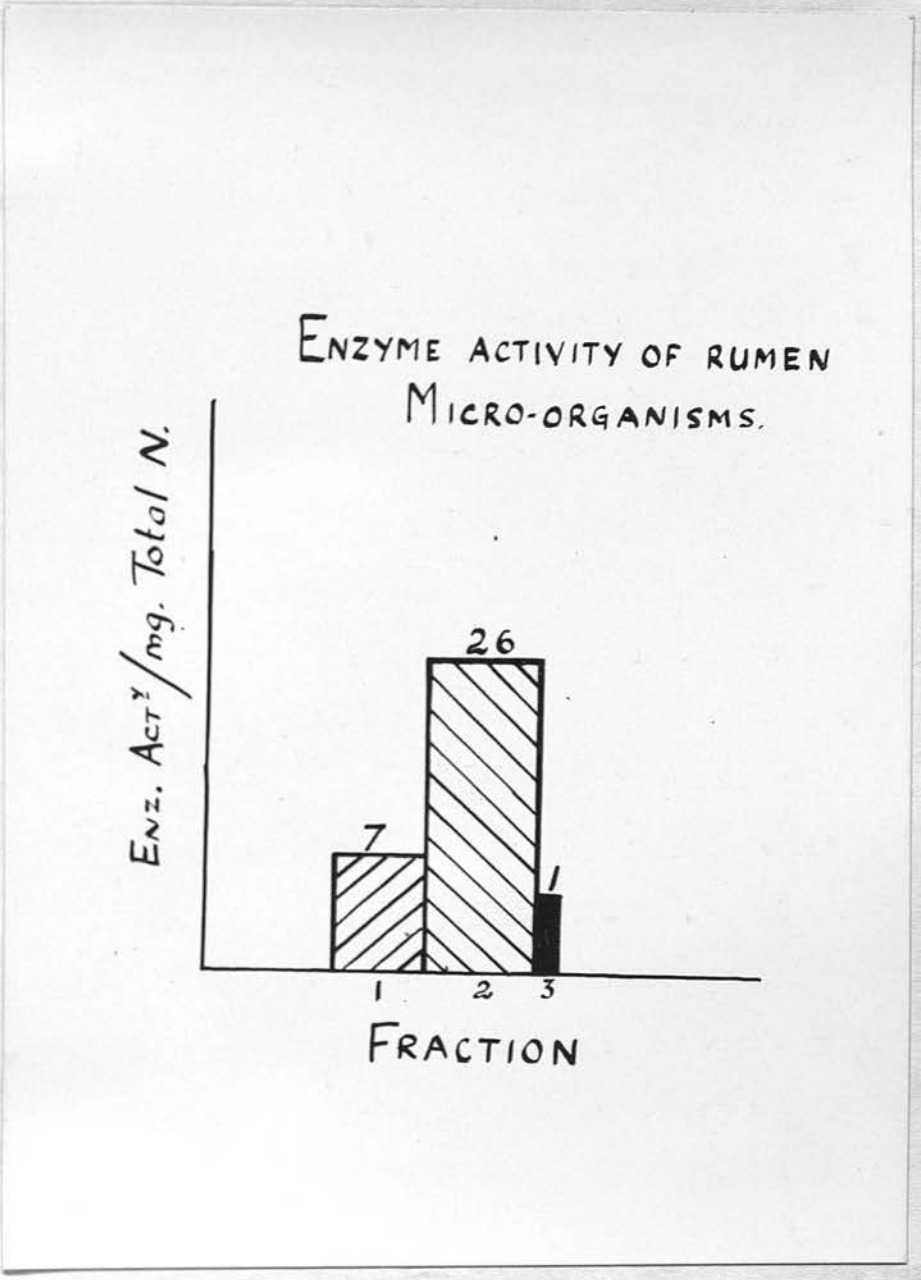
The various fractions as prepared by the above procedure were designated fractions (1), (2), (3) and (4). The sediments were resuspended in suitable volumes of water and their ability to decompose phenolphthalein- -d- glucuronide to liberate free phenolphthalein estimated by the method described before. Estimations of total N were also made on the various fractions. The final supernatant - fraction (4) - was devoid of enzyme activity. The results of these estimations are shown graphically in fig. (5).

The heights of the blocks represent the relative concentrations of the enzyme in the various fractions when expressed per unit of total N. The areas of the blocks show the relative total activities of the three fractions. The relative concentrations are in the ratio 3:8:2 and the total activities in the ratio 7:26:1. It is quite evident that the bulk of the activity and the greatest concentration of the enzyme per mg total N are in fraction (2).

D) Do the protozoa contain this enzyme?

By a process of repeated fractional

Fig. 5.


ENZYME ACTIVITY OF RUMEN MICRO-ORGANISMS.
ENZ. ACTY/mg. Total N.
26
7
1
1
2
3
FRACTION

sedimentation, it was possible to prepare a protozoal fraction which contained very few or no bacteria in it. The protozoal preparation was white in colour and consisted mainly of the larger sized species - holotrich ciliates and some large oligotrich ciliates. These protozoal suspensions were prepared starting from both strained rumen liquor and fraction (1). Although the initial starting fractions possessed high enzyme activity, the protozoal suspensions did not have any.

It would appear from these experiments that whatever activity was observed in fraction (1) was probably due to the presence of Quin's organisms and larger bacteria and not at any rate to the larger protozoa.

It was not found possible to prepare suspensions of the smaller protozoa, free of bacteria, by this method.

E) Is the enzyme activity associated with the organized activity of the cells?

Although it would appear that the enzyme being studied was a hydrolytic one, it was decided to study the effect of some measures, which would destroy or inhibit the organized activity of the cell as a whole, on the glucuronide decomposing activity of the micro-organisms. Two methods were examined.

In the first instance, the cells were dehydrated by treatment with acetone. An active fraction of the rumen micro-organisms was washed once or twice with water

to remove any colouring material and a thick suspension of the washed micro-organisms poured into 4 volumes of acetone with continuous stirring. The organisms coagulated and settled at the bottom of the vessel. After leaving the organisms in contact with the acetone for 10 mins., the coagulated mass was filtered at the pump, washed once with acetone and finally with ether. It was dried at the pump by suction till no trace of ether or acetone was noticeable. The preparation was finally dried in vacuo over $Ca\ Cl_2$. It was light brown in colour. If the initial washing of the micro-organisms was omitted, the final preparation tended to darken on exposure to the air - as the rumen liquor does, on standing exposed to air.

The enzyme activity of the organisms was studied both before and after the acetone treatment and also after keeping the acetone dried powder for some time in vacuo at room temperature. About 50-75% of the initial activity is retained in the acetone dried preparations and it would thus appear that the organized activity of the cell was not a necessary condition for the activity of the enzyme being studied. The 25-50% loss in activity may be due to denaturation of the enzyme protein occurring during the acetone treatment.

TABLE (1)

The effect of acetone treatment on the enzyme activity of micro-organisms

Nature of treatment	Activity per unit dry weight of organism (μg)		% activity retained
No acetone treatment	(a)	42	-
	(b)	26	
Acetone dried organisms soon after preparation	(a)	30	71
	(b)	15.6	60
Acetone dried organism kept in vacuo at R.T. for 23 days	(a)	16	38
	(b)	-	-

In a second series of experiments, the effect of adding a few drops of toluene to the incubation mixture was studied. If the enzyme were of an oxidation - reduction character, one would expect the addition of toluene to produce a decrease or complete inhibition of enzyme activity. No such effects were observed. In the tube containing toluene 102 μg of free phenolphthalein was liberated as opposed to 106 in the control tube. One is again led to conclude that the enzyme is probably a hydrolytic one and not one dependant on the organized activity of the cell as a whole.

F) Preparation of cell free enzyme

A cell free enzyme preparation was

obtained for the purpose of identifying and characterizing the enzyme. Preliminary studies had shown that the enzyme was most concentrated in fraction (2). This was therefore used as a source of the enzyme in all subsequent work.

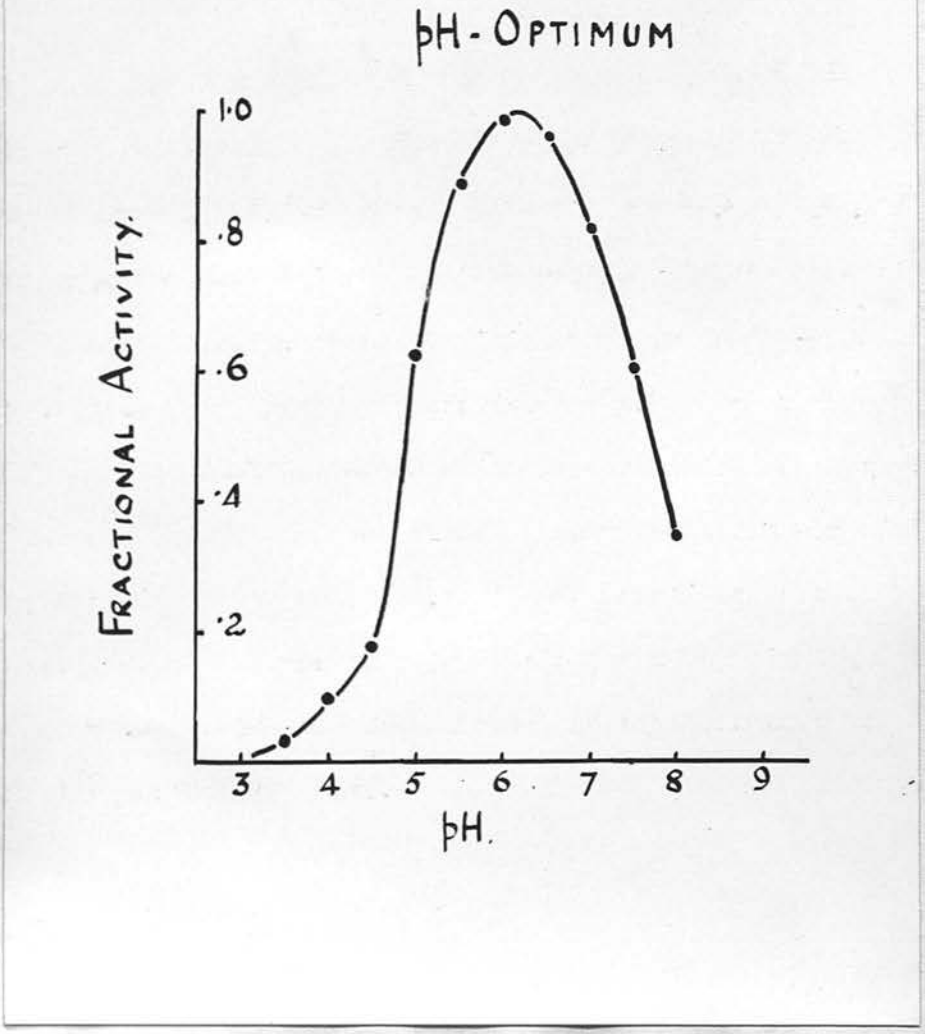
One method of preparing cell free enzyme extracts has been to extract dried preparations of organisms with suitable buffers. When this method was used with acetone dried organisms, it did not prove successful.

It has, however, been found possible to break down the cell walls of the organisms by shaking them at high speeds with fine glass beads and extracting the enzyme from the disintegrated cells with water. For this purpose, the Mickle tissue disintegrator, which is essentially a high speed shaker, and Ballotini beads (Grade 12 - Chance Bros. Ltd.) have proved very satisfactory. The organisms disintegrated in an aqueous medium were centrifuged at a high speed (8300 x g) when a cell free supernatant containing enzyme activity was obtained. It was sometimes necessary to centrifuge a second time to obtain the supernatant quite cell free. These cell free enzyme preparations were used in subsequent experiments.

G) pH - activity curve

The pH optimum for the activity of a cell free enzyme extract was studied. A mixed phosphate-citrate buffer was used over the pH range 3.0 - 8.0. Incubation tubes were set up containing 3.0 ml buffer, 0.5 ml

Fig. 6.



enzyme preparation and 0.5 ml substrate. A similar set of incubation mixtures was set up for pH measurements. In the enzyme blanks, water was substituted for substrate. At the end of an hour's incubation 4.0 ml of glycine - sodium carbonate buffer was added and the pH of each tube adjusted to that of the tube showing the maximum pH viz. 10.50. The solutions were all made up to 10 ml and the colours read on the spekker absorptiometer. The pH of the incubation mixtures was determined both before and after incubation and the average of the two values taken as the pH for that particular estimation. Incubation causes little change in pH ($\not>$ 0.4 pH unit). The results of three different experiments are expressed graphically in fig. (6). The activities are expressed as fractions of the maximum activity observed.

The pH optimum of the cell free enzyme extract is approximately 6.1 as is seen from the figure.

In the preliminary experiments, the estimation of enzyme activity was carried out at pH 4.5 in acetate buffer (0.1 M) as β-glucuronidase estimations were done at pH 4.5. All these experiments, however, were repeated at pH 6.1 using phosphate (0.1 M) buffer.

The enzyme activity of a cell free preparation was estimated in pure citrate, acetate and phosphate buffers at pH 6.1. The activities were identical and phosphate buffer was chosen for all further work with this enzyme.

TABLE (2)

Effect of various buffers on enzyme activity

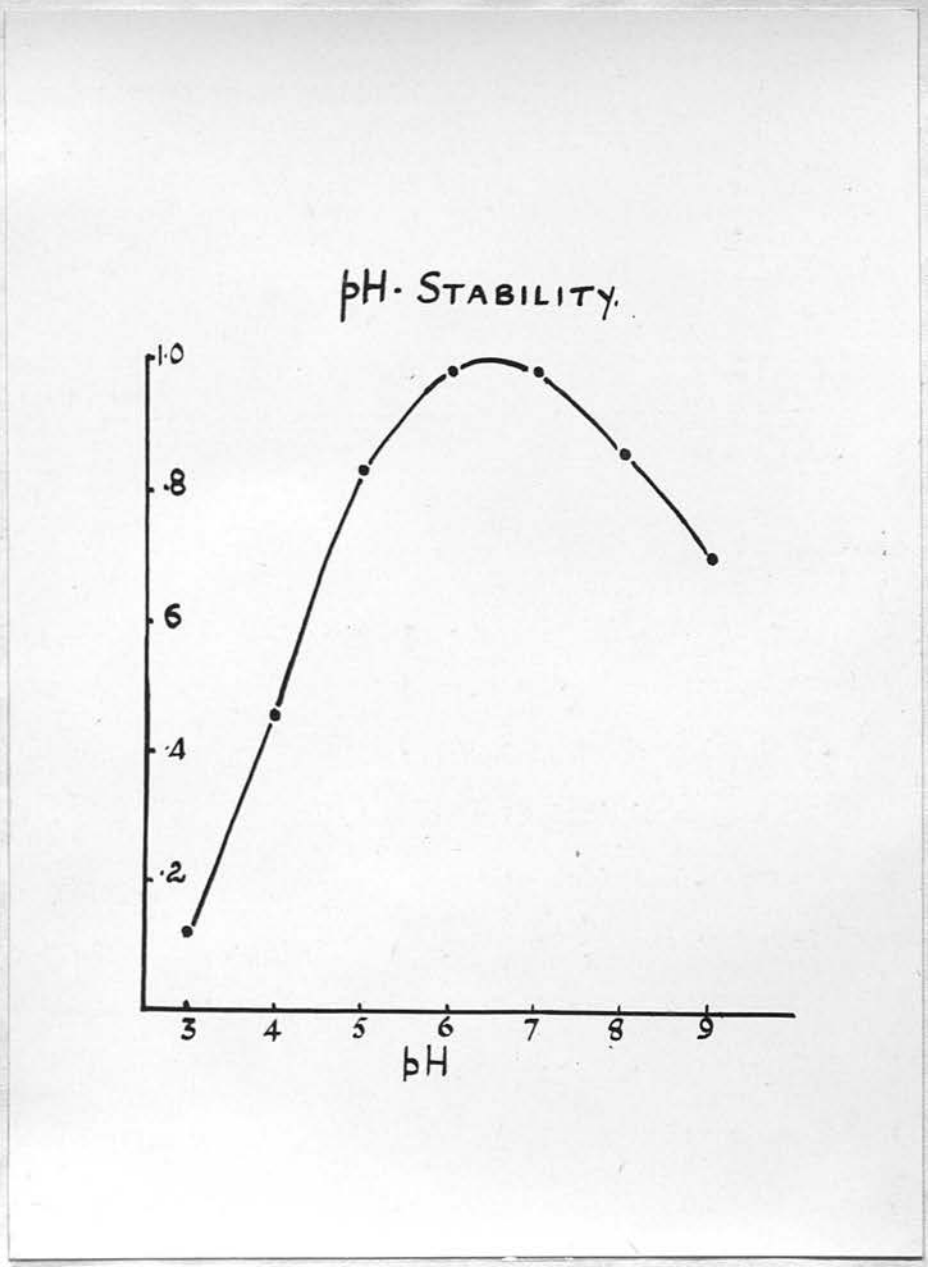
Buffer	Enzyme activity (μg phenolphthalein liberated)
Phosphate - citrate mixed buffer	24.6
Phosphate	25.8
Citrate	26.2
Acetate	25.0

H) pH stability curve

The pH of a cell free enzyme preparation was always about 6.3 - 6.5 before adjustment. In certain preliminary experiments, it was observed that when the pH of such an enzyme preparation was brought to 4.0 and then adjusted back to 6.1 a certain loss in enzyme activity took place. If the pH happened to fall to 2.0 even for a short time, the activity was completely lost and could not be restored by adjusting the pH back to 6.0.

Two experiments were conducted to find out how the enzyme activity reacted to pH changes over the range 3.0 - 9.0. An enzyme preparation was divided into a number of fractions. Each fraction was adjusted to a different pH (glass electrode). The enzyme solutions were then incubated at 37°C in the first experiment for 15 min. and in the second experiment for an hour. At the end of

Fig. 7.



the incubation, the pH of the enzyme solutions was adjusted to 6.1 and the solutions made up to known volumes. The activities of these solutions were measured in the usual manner. The results of the two experiments were almost identical and the results of the experiments in which the enzyme solutions were incubated for an hour are shown graphically (fig. 7). The activities are expressed as fractions of the maximum activity observed in the experiment. The enzyme appears to be most stable about pH 6.4; the activity being markedly destroyed below pH 5.0 and above pH 8.0.

I) Fractionation of the aqueous cell free enzyme extract by ammonium sulphate precipitation

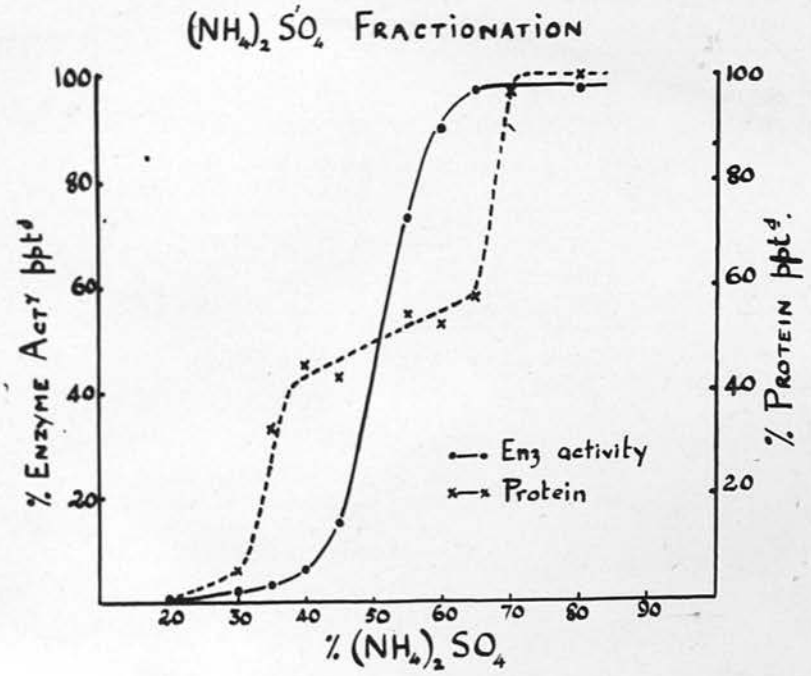
One of the methods of purifying an enzyme preparation is to precipitate out the enzyme protein from a solution by adding ammonium sulphate in increasing amounts, discarding the inactive protein fractions and obtaining the active enzyme fraction between certain limits of ammonium sulphate saturation. An aqueous cell free extract of the rumen micro-organisms would, in addition to the protein showing enzyme activity contain other inactive proteins. Some of these inactive proteins may be muco-proteins which tend to precipitate out of solution at an acid pH. Although making the pH of an aqueous solution of the glucuronide decomposing enzyme acid resulted in a partial loss in activity, it was found that the removal of inactive protein from the initial aqueous extract made possible the

subsequent concentration and purification of the enzyme protein.

Preliminary experiments showed that at pH 4.5 a considerable amount of inactive protein was removed. Subsequent to this treatment, any material precipitated up to 25% saturation with ammonium sulphate showed no enzyme activity. Neither did any material precipitating above 75% saturation. As a result of these preliminary studies, the following procedure was finally adopted for the further purification of the enzyme by ammonium sulphate fractionation.

The aqueous cell free extract (pH 6.3 - 6.5) was cooled to 0°C and the pH brought to 4.5 with acetate buffer. The mixture was centrifuged as quickly as possible in cooled centrifuge cups, and the pH of the supernatant immediately brought to 5.8 with the calculated amount of alkali and finally adjusted to 6.1. The solution was made 20% saturated with respect to ammonium sulphate by the addition of the required quantity of a saturated solution of ammonium sulphate adjusted to pH 6.1. The precipitate thrown down had no enzyme activity and was discarded. The supernatant was now made 75% saturated by the addition of more ammonium sulphate and the precipitate formed spun down on the centrifuge. This active enzyme fraction was redissolved in 0.1 M phosphate buffer pH 6.1. The enzyme solution was divided into 12 portions in tubes 1-12. Saturated ammonium sulphate solution at pH 6.1. was added to tubes

Fig. 8.


$(NH_4)_2SO_4$ Fractionation
% Enzyme Acty ppt'd
% Protein ppt'd.
Enz activity
Protein
% $(NH_4)_2SO_4$
20
30
40
50
60
70
80
90
100

1-11 to make the percentage ammonium sulphate saturation in the tubes range from 20% to 80% by 5% or 10% increments. Tube 12 was used as control. The protein precipitated in each tube was collected by centrifugation, dissolved in a known volume of water, dialysed overnight against running tap water and finally in distilled water. The enzyme activity and non dialysable N of each fraction were estimated. Fig. (8) shows the results of a typical experiment. Both total enzyme activity and total protein precipitated at the various percentage saturation with ammonium sulphate are expressed as percentages of the corresponding values obtained in the control tube (12).

It is evident from the graph that more than 90% of the enzyme activity is precipitated between 40 and 65% saturation with ammonium sulphate. In terms of protein N, this procedure results in a seven-fold concentration of the enzyme.

J) Time - activity curve

Experiments were conducted in which a partially purified enzyme preparation was incubated with 0.0125 M phenolphthalein glucuronide at pH 6.1 for various times. The results of these experiments are shown in fig. (9). It is quite clear that under the conditions used for the assay of enzyme activity, the amount of hydrolysis caused during an hour's incubation may be taken as a measure of the maximum velocity of hydrolysis with the

Fig. 9.

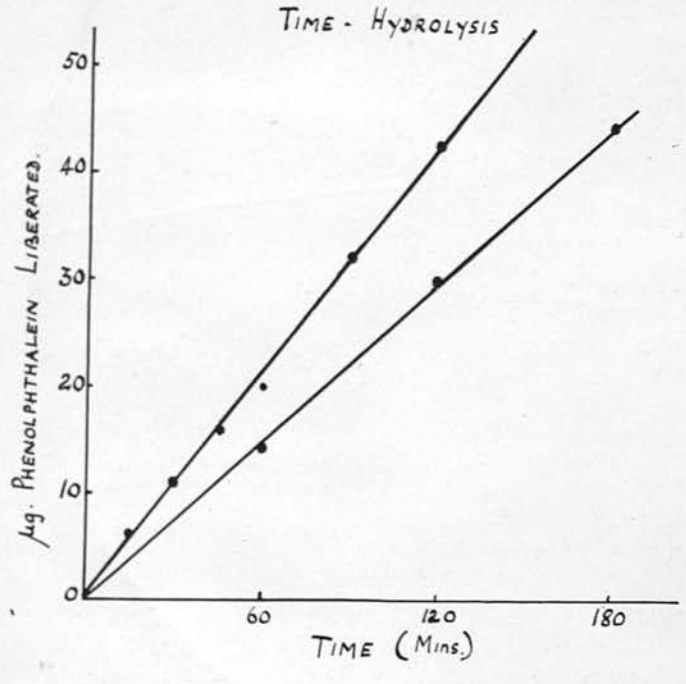
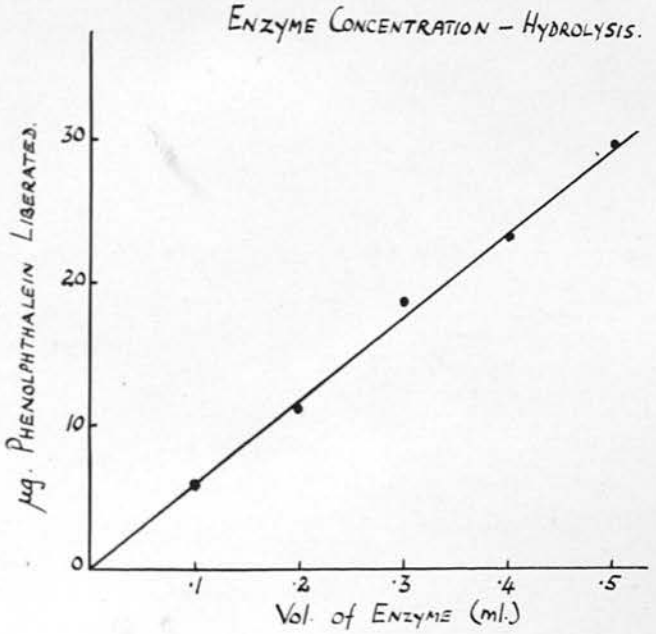

TIME - HYDROLYSIS
µg. PHENOLPHTHALEIN LIBERATED.
0
10
20
30
40
50
60
120
180
TIME (Mins.)

Fig. 10.


Enzyme Concentration – Hydrolysis.
μg. Phenolphthalein Liberated.
30
20
10
0
·1
·2
·3
·4
·5
Vol. of Enzyme (ml.)

enzyme preparation and therefore as a measure of the enzyme activity.

K) Enzyme concentration - activity curve

Fig. (10) shows the effect of varying the concentration of the enzyme preparation on the amount of hydrolysis obtained when the enzyme was incubated for an hour with 0.0125 M phenolphthalein glucuronide at pH 6.1 in phosphate buffer. It is quite evident from the figure that under the conditions used the relationship between velocity of hydrolysis and enzyme concentration obeys the equation

$$v = ke$$

Where k = constant and e = enzyme concentration.

L) The effect of dialysis on the enzyme activity of an aqueous cell free preparation

If a dialysable component formed part of the enzyme system responsible for the breakdown of phenolphthalein-β-d-glucuronide, to give free phenolphthalein, the activity of the preparation should be reduced considerably if not lost entirely on dialysis. A known volume of an enzyme preparation was dialysed for 20 hours against running tap water. As control a quantity of the same enzyme preparation was placed in a stoppered glass vessel in the same container of water in which the dialysis sac was placed. At the end of the dialysis, the two enzyme fractions were estimated for their activity. The undialysed enzyme had an activity of 160 g/ml while the dialysed enzyme had an

activity of 142 µg/ml. Only a small drop in activity (12%) was observed in the dialysed enzyme preparation and it is quite reasonable to conclude that no easily dialysable moiety forms part of the enzyme system responsible for the production of free phenolphthalein from its glucuronide.

M) Is a heat stable component associated with the enzyme?

If a heat stable molecule was associated with the enzyme, then the addition of a boiled aqueous enzyme preparation to an unboiled preparation of the enzyme might produce an effect on the observed enzyme activity. Three sets of incubation mixtures were made up. The first consisted of

2.5 ml buffer

0.5 ml unboiled enzyme preparation

0.5 ml boiled enzyme preparation

0.5 ml substrate solution

In the second, the boiled enzyme preparation was replaced by water and in the third, the unboiled enzyme preparation was replaced by water. Appropriate enzyme controls were also set up. Almost identical hydrolyses were observed in sets (1) and (2) and no hydrolysis in set (3), showing that

(a) boiling the enzyme solution had caused destruction of the enzyme.

(b) if a heat stable component was associated with the enzyme activity it was already present in sufficient amounts in the enzyme preparation,

or (c) if the second component was not present in optimum quantities it was heat labile.

TABLE (3)

The effect of adding boiled aqueous cell free enzyme extract on the hydrolysis caused by the unboiled aqueous cell free extract

Nature of enzyme preparation	µg phenolphthalein liberated in hydrolysis tube
Boiled + unboiled aqueous preparations of enzyme	46.0
Boiled aqueous preparation	0.5
Unboiled aqueous preparation	44.0

N) The inhibitory effect of certain substances on the enzyme activity

Since the enzyme was capable of decomposing phenolphthalein glucuronide, it was thought that the enzyme might bear some resemblance to animal β-glucuronidase and consequently be inhibited by saccharic acid. The effect of saccharic acid and a few other substances were tested on the enzyme activity of a cell free aqueous preparation. Saccharic acid was found to have a small inhibitory effect while the following substances had no effect at all. d-glucose (0.01 M), d-galactose (0.01 M), d-mannose (0.01 M), d-arabinose (0.01 M), l-arabinose (0.01 M) d-ribose (0.01 M) d-xylose (0.01 M), glucurone (0.01 M), galacturonic acid (0.0015 M), mucic acid (0.01 M).

Saccharic acid has only a small inhibitory effect in a concentration which causes 100% inhibition of mouse liver β-glucuronidase. It would appear that the enzyme being studied was different from animal β-glucuronidase.

TABLE (4)

Inhibition of enzyme activity by saccharic acid

Nature of enzyme preparation	Concentration of saccharic acid (M)	% Inhibition
Ammonium sulphate precipitated fraction	0.1	44
Ammonium sulphate precipitated fraction	0.01	8
Suspension of micro organisms	0.01	0

o) Recovery of added phenolphthalein after incubation with whole organisms and cell free enzyme preparations

A few experiments were done in which the percentage recoveries of phenolphthalein added to suspensions of micro-organisms causing hydrolysis of phenolphthalein glucuronide or to cell free enzyme preparations were estimated. There was the possibility of the intact organism or enzyme preparations destroying phenolphthalein during the incubation. Results are shown in table (5).

TABLE (5)

Percentage recovery of added phenolphthalein

Nature of enzyme preparation	µg phenolphthalein recovered		% Recovery
	In control	In presence of enzyme prep^{n}	
Aqueous suspension of micro-organisms	29.0	29.6	102
Cell free aqueous extract	28.5	28.5	100

The percentage recoveries obtained show that no destruction of free phenolphthalein occurs during the incubation.

P) Substrate concentration - activity curve

The effect of varying the substrate concentration on the amount of hydrolysis caused by an enzyme preparation was studied. A partially purified enzyme preparation was used. The results of a typical experiment are expressed graphically in figs. (11) and (12).

In fig. (11) the activity observed has been plotted against log S - the substrate concentration. Inhibition by excess substrate occurs beyond a substrate concentration of 0.01 M while maximum activity is obtained with 0.001 M substrate concentration.

For an enzyme which is inhibited by excess substrate, the velocity of the enzyme reaction is

Fig. 11.

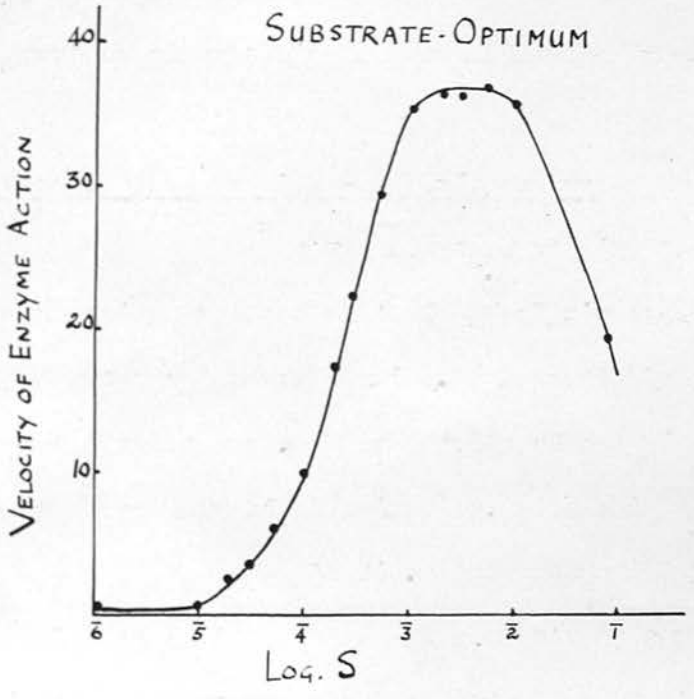
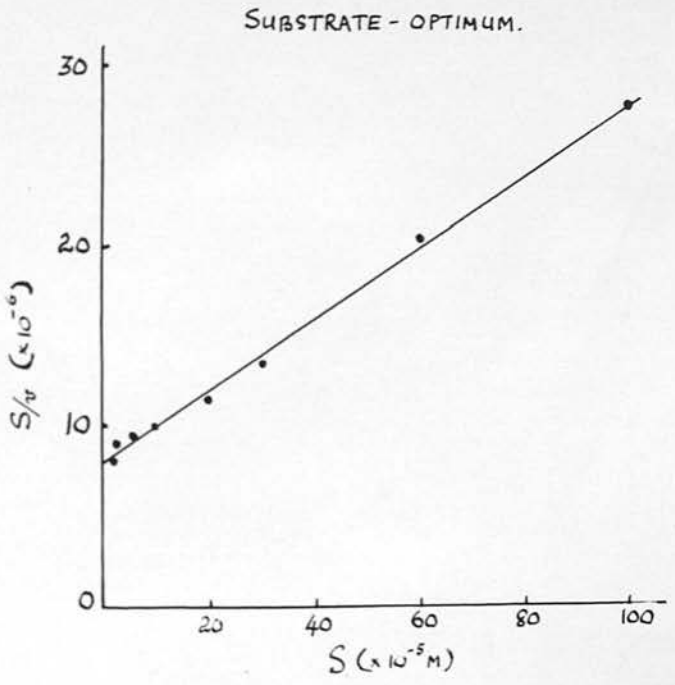

SUBSTRATE-OPTIMUM
VELOCITY OF ENZYME ACTION
40
30
20
10
$\bar{6}$
$\bar{5}$
$\bar{4}$
$\bar{3}$
$\bar{2}$
$\bar{1}$
LOG. S

Fig. 12.



given by the equation:- (Lineweaver & Burk 1934).

$$v = \frac{V_{max}\ S}{S + K_s + \frac{S^n}{K_2}} \qquad \text{——} \quad (1)$$

The reaction may be represented as follows:-

$$E + S \rightleftharpoons ES \quad \text{(active)}$$

$$ES + (N-1)\ S \rightleftharpoons ES_n \quad \text{(inactive)}$$

In eqn (1) v = observed velocity

V_{max} = maximum velocity

S = substrate concentration

K_s = dissociation constant of ES

K_2 = dissociation constant $\frac{ES\ \ S^{n-1}}{ES_n}$

Equation (1) may be written:-

$$\frac{S}{v} = \frac{K_s}{V_{max}} + \frac{1}{V_{max}}\left(S + \frac{S^n}{K_2}\right) \qquad \text{——} \quad (2)$$

If $\frac{S}{v}$ is plotted against S, then at low concentrations of S the graph is a straight line. From this part of the graph a value for V_{max} and K_s may be obtained from the slope and intercept.

In fig. (12) $\frac{S}{v}$ has been plotted against S and from the graph values for K_s and V_{max} have been obtained

$$K_s = 4.04 \times 10^{-4}\ M$$

$$V_{max} = 50.5$$

An attempt was made to evaluate 'n'. It appears to have a value 2, but more experiments have to be carried out before a value of 2 can be assigned to 'n' with any certainty.

DISCUSSION

Although the subject of ruminant digestion has received considerable attention in the past and attempts have been made to follow the process of digestion occurring in the rumen, the presence of a large variety of micro-organisms present in the rumen has been a great handicap. As yet, no complete picture of the type of organisms present in the rumen is available. This may be due largely to the fact that the nature of the diet given to a sheep influences the type of organism present in the rumen. Different diets would thus give rise to different pictures of the types of organisms present in the rumen. An attempt to isolate the organisms in a pure culture and then study their metabolism *in vitro* in an effort to find out the role they play in ruminant digestion appears to be a well nigh impossible task at present. Some workers have therefore confined themselves to a microscopic study of rumen contents with a view to gathering information about the micro-organisms which act on cellulose and other large food particles. Others have concentrated their attention on identifying the end products of digestion absorbed from the rumen, and still others are concentrating their attention on studying the nature of

substances accumulating in the micro-organisms during the digestion of foodstuffs in the rumen.

As a result of all this work, a somewhat incomplete picture of what happens in the rumen may be painted. Food entering the rumen is broken down by the micro-organisms into simpler substances. Part of the food is oxidised to provide energy for the growth and maintenance of the organisms. During this process, CO_2 CH_4 and volatile fatty acids are liberated. These fatty acids, acetic, propionic, butyric and others and also lactic acid are absorbed from the rumen and used by the animal. The rest of the food which is converted into starch-like cellular material in the micro-organisms becomes available to the animal as a result of the death of the micro-organisms in the rumen, or when the micro-organisms are broken down in the abomasum. Whether storage of polysaccharides in the micro-organism is a necessary intermediate step or not in ruminant nutrition is a point of much debate.

Considerable attention has been paid to the nature of the end products of digestion appearing in the rumen. Very little work, if any, has been done on the initial stages of digestion. It was felt that a study of the hydrolytic enzymes present in the rumen might throw some light on the problem. Instead of studying the enzyme system from isolated pure cultures, it was thought it would be better to treat all the micro-organisms present as a single entity and study the hydrolytic enzymes present in it.

From the nature of the polysaccharides present in normal ruminant foodstuffs it was felt that a β-glucuronide decomposing enzyme would very likely be present in the rumen, and consequently a search was made for it. The use of a simple soluble molecule with the characteristic β-linkage is a great asset in preliminary studies of the enzyme. Nevertheless, studies with the normal substrates present in the foodstuffs have to be carried out with the enzyme once it has been fairly well characterized. In the preliminary studies reported, some of the characteristics of the enzyme have been established. Further points requiring immediate attention are:-

1). Study of enzyme specificity with highly purified preparations. It should then be possible to give the enzyme a name.

2). Complete identification of the fission products from phenolphthalein glucuronide.

3). A study of its action on possible substrates in ruminant foodstuffs.

4). Identification of the organism or organisms containing this enzyme. The histochemical methods for β-glucuronidase may be of some assistance in this connection.

Work along these lines is in progress at the moment.

The little evidence available would appear to suggest that the enzyme is different from animal

glucuronidase. It has a different pH optimum, 6.1 instead of 5.2 and 4.5. It is precipitated between 40-65% ammonium sulphate saturation while β-glucuronidase is completely precipitated between 30-44% saturation and finally, while 10^{-2}M saccharic acid completely inhibits β-glucuronidase activity it has only very slight inhibitory effect on this enzyme.

In view of the fact that specificity tests have not been completed, the function of this enzyme in the rumen is one for speculation. It seems possible, however, that it enters into the breakdown of the polyuronides into simpler uronic acids to make them available to the animal either directly or via the micro-organisms.

SUMMARY

1). A glucuronide decomposing enzyme has been found in the "medium" sized micro-organisms present in the rumen of the sheep.

2). It does not appear to be present in the large protozoa.

3). The enzyme activity does not depend on the organized activity of the cell as a whole and the enzyme appears to be a purely hydrolytic enzyme.

4). A cell free enzyme preparation has been obtained from the active micro-organism by disintegrating the cells with Ballotini beads, and subsequent centrifugation at high speed to obtain a clear active supernatant.

5). The pH optimum of such a preparation is 6.1.

6). It is stable at a pH of 6.3 - 6.5 and is markedly destroyed below pH 5.0 or above pH 8.0.

7). No dialysable component or heat stable co-enzyme appears to be involved in the enzyme reaction.

8). The active enzyme protein is almost completely precipitated between 40 and 65% saturation with ammonium sulphate.

9). The enzyme is only very slightly inhibited by saccharic acid.

10). A value for K_s - the Michaelis Menten constant for the enzyme complex with phenolphthalein glucuronide has been obtained. viz: - 4.04×10^{-4} M.

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The Inhibition of β-Glucuronidase by Saccharic Acid and the Role of the Enzyme in Glucuronide Synthesis

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view of the relationship shown to exist between β-glucuronidase activity of a tissue and its state growth (Levvy, Kerr & Campbell, 1948), it was sidered important to find a specific inhibitor for enzyme. It has been suggested (Fishman, 1940), hout any direct evidence, that β-glucuronidase esponsible for the formation of glucuronides in body. The use of an inhibitor for glucuronidase esting this hypothesis forms an obvious first step vards elucidating the physiological function of the yme.

A variety of substances have been examined for ir effect on the hydrolysis of phenylglucuronide by β-glucuronidase. Of those which caused inhibition, by far the most effective was D-glucosaccharic acid, and this compound was examined for its action on glucuronide synthesis by liver slices and on growth processes in the mouse.

EXPERIMENTS AND RESULTS

Determination of β-glucuronidase. The hydrolysis of phenylglucuronide by mouse-liver or kidney glucuronidase preparations was measured by the procedure of Kerr, Graham & Levvy (1948). In testing substances for a possible inhibitory action on the enzyme, incubation mixtures were made up as follows: 0·4 ml. enzyme preparation, 0·2 ml.

Table 1. *Inhibition of β-glucuronidase* in vitro (0·015 M-*phenylglucuronide*)

Compound	Concentration (10^{-4} M)	Phenol liberated: In controls (μg.)	Phenol liberated: In presence of inhibitor (μg.)	Inhibition (%)	Enzyme preparation
Saccharic acid	150	32·1	3·3	90	Liver
	150	25·4	4·2	84	,,
	150	17·1	3·2	81	,,
	50	39·1	8·0	80	,,
	50	32·7	6·5	80	,,
	50	27·0	6·5	76	,,
	50	25·5	7·9	69	,,
	50	21·2	3·2	85	,,
	50	16·5	5·8	65	,,
	50	20·5	4·6	78	Liver *A*
	50	22·8	3·4	85	Liver *B*
	50	22·5	0·5	98	Crude liver
	50	11·8	3·8	68	Kidney
	50	21·2	6·5	70	,,
	50	17·0	5·7	67	Crude kidney
Mucic acid	75	30·2	23·6	22	Liver
D-Gluconic acid	150	37·4	28·8	23	,,
D-Glucurone*	300	40·0	0	100	,,
	150	35·3	12·1	66	,,
	38	55·2	32·4	41	,,
	10	55·2	45·2	18	,,
	3·3	55·2	51·2	7	,,
L-Malic acid†	150	23·7	14·9	37	,,
DL-Malic acid	300	32·9	18·6	44	,,
	150	30·2	23·5	22	,,
Phlorrhizin‡	3	16·3	13·6	17	,,
	1·5	16·3	16·8	−3	,,
Vanillin‡	7·5	39·4	28·8	27	,,

* Interferes in colour reaction for phenol. Results are corrected for interference.
† The naturally occurring isomer, commonly called laevorotatory malic acid.
‡ Gives colour with phenol reagent. Results are corrected for this colour.

0·1 M-citrate buffer, 0·1 ml. 0·12 M-phenylglucuronide, 0·1 ml. inhibitor solution. In controls, water was substituted for the inhibitor solution. Buffer, substrate and inhibitor solutions were, as a rule, adjusted to pH 5·2 (glass electrode). In experiments in which the two glucuronidase fractions in mouse liver were separated (Kerr, Campbell & Levvy, 1949; Mills, 1948), however, hydrolysis with fraction *A* was carried out at pH 4·5 instead of 5·2. Occasionally, preliminary purification of the enzyme was omitted, and the crude liver or kidney homogenate was used for hydrolysis. Results are expressed as μg. of phenol liberated in 1 hr. at 37°.

Measurement of glucuronide synthesis. The conjugation of *o*-aminophenol with glucuronic acid was followed by the method of Levvy & Storey (1949). After removal of protein with a mixture of trichloroacetic acid and phosphate buffer, the glucuronide was diazotized and coupled with naphthylethylenediamine. At the pH selected for colour development, free *o*-aminophenol in comparatively large amounts did not interfere. To measure the synthetic activity of mouse-liver slices, they were shaken in sulphate-free bicarbonate Ringer solution, containing 0·02 M-lactate, 0·001 M-ascorbic acid and 0·0025% *o*-aminophenol, at 37° for 1 hr. in an atmosphere of 5% CO_2 in O_2. Results are expressed as μg. *o*-aminophenol conjugated/g. dry wt. of tissue in 1 hr.

Inhibition of β-glucuronidase in vitro

Nearly fifty substances were examined for their effect on β-glucuronidase *in vitro*. Those which caused a fall in the activity of the enzyme are listed in Table 1. The most effective was D-glucosaccharic acid, and Fig. 1 shows the percentage inhibition produced by varying concentrations of this compound in three experiments with liver glucuronidase. It can be seen that 50% inhibition was obtained with 2×10^{-4} M-saccharate, and practically complete inhibition with less than 10^{-2} M (substrate concentration 0·015 M). From results given in Table 1, it appears that the inhibitory action of saccharate was independent of the following factors: the source of the enzyme, the activity of the preparation, the glucuronidase fraction present, and the degree of purity of the preparation.

Of other compounds listed in Table 1, three were closely related to saccharic acid (mucic, gluconic and glucuronic acids), but were much less efficient as inhibitors of β-glucuronidase. Glucuronic acid (D-glucurone) interfered in the determination of phenol liberated from phenylglucuronide. In the absence of phenol, glucuronic acid was without effect on the Folin-Ciocalteu reagent, but in the presence of phenol it apparently gave the colour reaction. This effect was independent of the phenol concentration. The figures shown in Table 1 for the inhibitory action of glucuronic acid on glucuronidase have been corrected for interference in the colour reaction, and

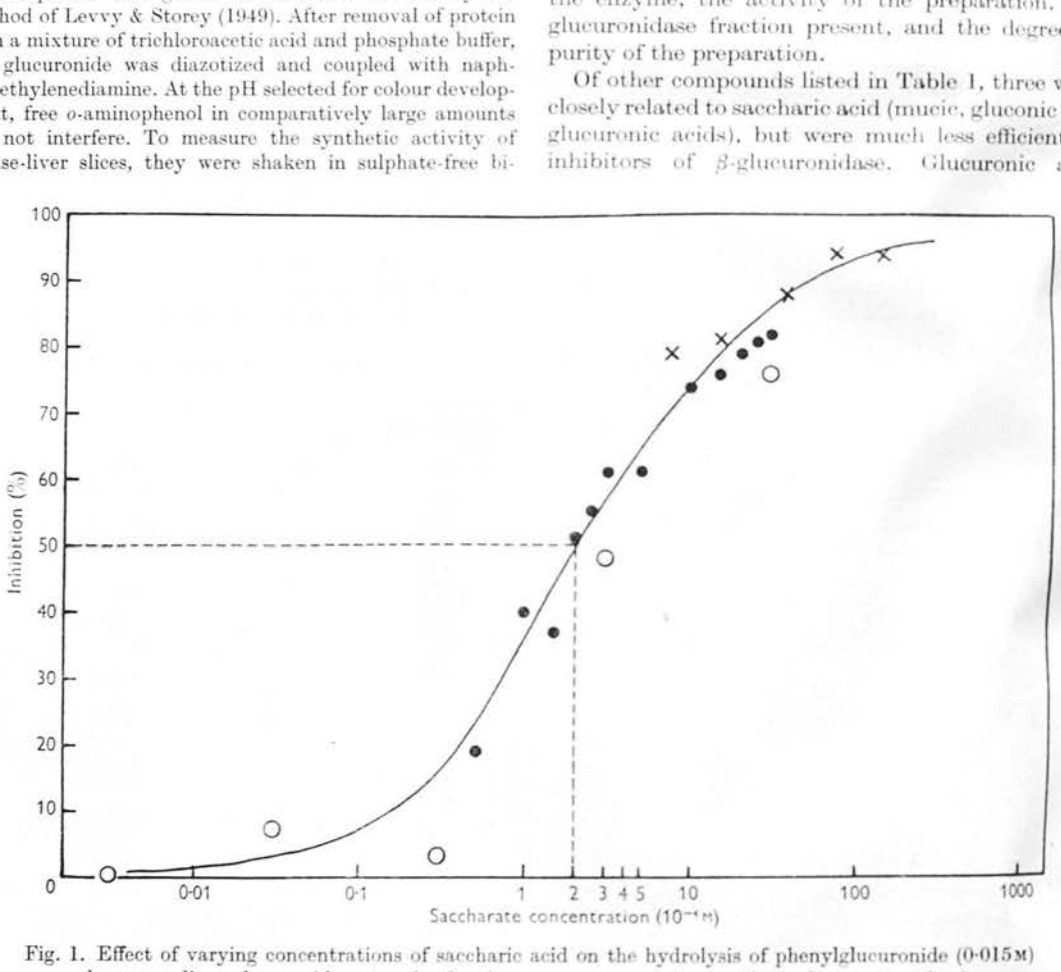

Fig. 1. Effect of varying concentrations of saccharic acid on the hydrolysis of phenylglucuronide (0·015 M) by mouse-liver glucuronidase (results for three separate experiments shown by ○, ● and ×).

sted i considered reliable. Correction of the hydrolysis
ccharic es was also necessary in the case of phlorrhizin
ibitio vanillin which gave the colour reaction directly.
s com r correction, the results suggested that both
nidase pounds slightly inhibited β-glucuronidase. L-
ed wit ic acid in high concentration had an inhibitory
lete in on the enzyme which entirely accounted for
centra effects produced by the racemic acid. A com-
e 1, itive study of the tartaric acids might provide
ate wa esting information regarding configurational
urce irements for glucuronidase inhibition. Un-
n. th mately, only L-tartaric acid* was available, and
gree o had no effect on the enzyme (see below).

e wer e following substances had no apparent effect on the
nic an olysis of phenylglucuronide by β-glucuronidase in the
ient e entrations shown: β-phenyl-D-glucoside (0·015 M),
c aci thyl-D-glucoside (0·015 M), α-methyl-D-mannoside
3 M), β-methyl-D-glucoheptoside (0·003 M), β-methyl-D-
side (0·003 M), α-methyl-D-galactoside (0·003 M), gum
ic (0·15 %), degraded egg-plum gum (0·05 %), pyromucic
(0·015 M), sorbic acid (0·02 M), oxalic acid (0·015 M),
nic acid (0·015 M), succinic acid (0·015 M), glutaric acid
5 M), maleic acid (0·015 M), L-tartaric acid (0·015 M),
ic acid (0·015 M), ouabain (0·0015 M), digitonin (0·0015 M),
ane (0·015 M), phenylurethane (0·015 M), nitroso-*N*-
ylurethane (0·015 M), heparin (6·6 Toronto units/ml.),
apyridine (0·00015 M), inositol (0·015 M), piperonal
5 M), *n*-hexyl alcohol (0·015 M), *NN*-di-(2-chloro-
l)-aniline (0·02 M), 2′-methyl-4-dimethylaminostilbene
(M), NaF (0·015 M), Na_2SO_4 (0·03 M).

e following compounds gave colours with the phenol
nt, but, when correction was made for this, they were
rently without effect on β-glucuronidase in the con-
rations shown: salicin (0·015 M), thiourea (0·0015 M),
ne (0·0001 M), ascorbic acid (0·00075 M), oestrone
05 M), colchicine (0·0001 M).

e following compounds interfered too badly in the
r reaction to be tested with β-glucuronidase: sodium
ω-bromoacetophenone, phenylarsenoxide, ethyl cyano-
te, ethane-1:2-dithiol.

The action of saccharic acid on β-glucuronidase

rom its similarity in structure to glucuronic acid,
would expect saccharic acid to act competitively
hibiting glucuronidase. That the inhibition was
rsible was shown by precipitating the enzyme
n 0·03 M-saccharate solution with an equal volume
turated $(NH_4)_2SO_4$ solution. After one precipita-
, the activity was 70 % of that shown by a control
ple of the enzyme. After dissolving in water and
ecipitating, the activity was as great as in the
rol.

he normal substrate-activity curve for the
rolysis of phenylglucuronide by mouse-liver
uronidase has been studied by Kerr *et al.* (1948;
also Kerr *et. al.* 1949). An approximate value
·0035 M was obtained for K_m, the concentration
giving half the maximum velocity of hydrolysis. The maximum was usually reached with 0·015 M-substrate. Inhibition by excess substrate was pronounced.

Fig. 2 shows the effect of increasing the concentration of phenylglucuronide on its initial rate of hydrolysis in presence of 2×10^{-4} M-saccharate. Results are expressed in terms of the relative activity, where hydrolysis of 0·015 M-phenylglucuronide in absence of inhibitor is taken as unity. The points show averages for two representative experiments, one with liver glucuronidase fraction *A* and the other with fraction *B*. There was no appreciable difference in the results for the two fractions. It is clear that

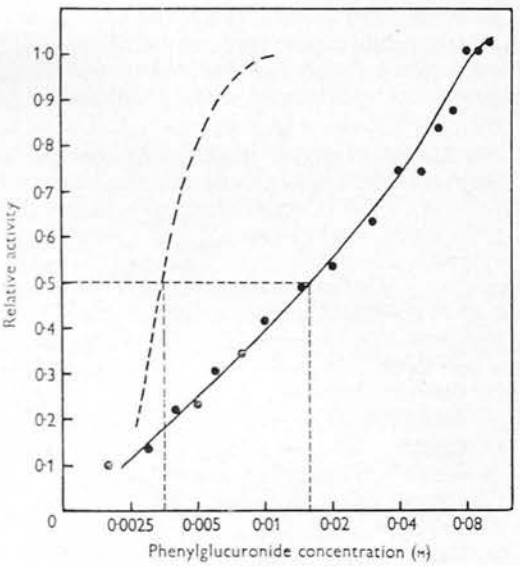

Fig. 2. Effect of varying concentrations of phenylglucuronide on its hydrolysis by mouse-liver glucuronidase in presence of 2×10^{-4} M-saccharate (●——●). Results expressed as fractions of the maximum activity observed in absence of inhibitor. Substrate-activity curve in absence of inhibitor (Kerr *et al.* 1948) shown by broken line.

saccharate acted competitively since the effect decreased with increasing substrate concentration till at 0·08 M-phenylglucuronide the activity of the enzyme was fully restored.

From Figs. 1 and 2, it can be seen that, in presence of a saccharate concentration [I] of 2×10^{-4} M, half the maximum enzyme activity was reached with a substrate concentration of 0·015 M. The latter figure can be designated K'_m, and K_i, the dissociation constant of the enzyme-inhibitor complex, can be calculated from the equation $K_i = [I] . K_m/(K'_m - K_m)$ (Lineweaver & Burk, 1934). Using the value for K_m given by Kerr *et al.* (1948), $K_i = 6 \times 10^{-5}$ M. It should be stressed that, presumably owing to the presence of impurities, K_m can vary from enzyme preparation to preparation by as much as 50 % in absence

* The naturally occurring isomer, commonly called dextrorotatory tartaric acid.

of added inhibitor. This is associated with variations in the substrate concentrations at which maximum activity is reached and inhibition by excess substrate becomes marked. The value for K_i is not materially altered since K'_m varies with K_m. Attempts to determine K_i by the method of Hunter & Downs (1945), in which knowledge of K_m is not required, were unsuccessful.

The effect of saccharic acid on glucuronide synthesis by mouse-liver slices

Table 2 shows the effect of saccharic acid in varying concentration on the formation of *o*-aminophenylglucuronide by mouse-liver slices. Each figure is an average for a determination done in quadruplicate, as recommended by Levvy & Storey (1949). These authors found the standard deviation of a single observation from the mean to amount to 17 %

Table 2. *Action of dicarboxylic acids on glucuronide synthesis by mouse-liver slices*

Exp. no.	Compound	Concentration (10^{-4}M)	*o*-Aminophenol conjugated/g. dry wt. (μg.)	Difference from control (%)
1	Control	—	210	
	Saccharate	8	190	−10
2	Control	—	200	
	Saccharate	8	170	−15
3	Control	—	460	
	Saccharate	50	500	+ 9
4	Control	—	390	
	Saccharate	50	300	−23
5	Control	—	620	
	Saccharate	50	540	−13
	Saccharate	100	540	−13
6	Control	—	840	
	Saccharate	100	580	−31
	Saccharate	170	550	−34
7	Control	—	340	
	Saccharate	100	270	−20
	Maleate	100	270	−20
8	Control	—	780	
	Saccharate	100	720	− 8
	Succinate	100	660	−15

in their procedure. The standard error for a figure based on four results is thus 10 %. Differences in synthetic activity between saccharate-treated slices and control slices from the same animal approached significance in only one experiment (no. 6). Taking the results as a whole, however, synthesis in presence of saccharate tended to be slightly less than in its absence. This effect was non-specific since it was also seen in experiments with succinic and maleic acids. These acids have no action on β-glucuronidase (see p. 601). All three dicarboxylic acids studied were added as solutions brought to neutrality with potassium hydroxide (glass electrode).

Experiments were done to show that saccharic acid can inhibit hydrolysis of *o*-aminophenyl glucuronide by β-glucuronidase. The final substrate concentration was arbitrarily fixed at 0·13 % and the pH at 4·5 (citrate buffer). After 2 hr. incubation at 38° with mouse-liver glucuronidase, 11 % hydrolysis of the glucuronide was observed in absence of saccharate. In presence of 10^{-3}M-saccharate, the hydrolysis was 0·7 %. When the experiment was repeated with another enzyme preparation, the hydrolysis in absence of saccharate was 17 % and in its presence 3·6 %.

Penetration of saccharic acid into the cell

Attempts were made to show inhibition of β-glucuronidase in the intact cell by saccharic acid. In the first experiment, mouse-liver slices of known weight from two animals were shaken in sulphate-free bicarbonate Ringer solution containing 0·01M saccharate for 90 min. at 37°. At the end of the period, the slices were removed, washed in three changes of distilled water, and homogenized. Inactive protein was precipitated by incubation of the homogenate for 30 min. at pH 5·2. Without further purification, the supernatant was examined for β-glucuronidase activity. The activity in terms of μg. phenol liberated at 37° in 1 hr. by 1 g. liver was 220, compared with 334 for control slices from the same two animals put through the procedure in absence of saccharate. Unless saccharate were strongly adsorbed on the surface of the slices, it would appear that an appreciable amount penetrated the cells. On the assumption that the saccharate concentration within the slices rose to 0·01M, the inhibition expected was of the order of 75 %.

In other experiments, the enzyme preparation in the incubation mixture used in the assay of glucuronidase activity was replaced by mouse-liver slices of known weight. The results had little quantitative value as the 'enzyme blank' was variable and high, but they suggested that some hydrolysis of phenyl glucuronide took place and that this process was strongly inhibited by 0·015M-saccharate.

The effect of saccharate on the oxygen uptake and anaerobic glycolysis of mouse-liver slices

Saccharate in a concentration of 0·014M had no effect either on the oxygen uptake or on the anaerobic glycolysis of mouse-liver slices as measured in the Warburg apparatus. The Ringer solutions of Krebs & Henseleit (1932) were used, the O_2 consumption being determined in phosphate Ringer and an atmosphere of O_2, and the CO_2 output in bicarbonate Ringer and an atmosphere of 5 % CO_2 in N_2.

The action of saccharic acid on growth processes in the mouse

A compound that inhibits β-glucuronidase *in vitro* would be hardly expected to arrest whatever mechanism is responsible for the increase in the activity of the enzyme normally observed *in vivo* when a tissue is stimulated to rapid growth. If, however, glucuronidase plays an essential part at some stage in the growth process, administration of an inhibitor might modify the process at that stage. This possibility was examined with saccharic acid. When large doses were administered to mice, saccharic acid was apparently without effect on liver regeneration following damage and on growth in infant mice. Figures for glucuronidase activity, the weights of single organs or of the whole animal, and the histological picture were invariably identical with those observed in appropriate controls. It should be pointed out that during the preparation of the enzyme for assay it would be freed from any saccharic acid which might have been present in the original tissue.

Saccharic acid given at frequent intervals by subcutaneous injection of neutralized solutions of the potassium hydrogen salt in doses totalling up to 2 g./kg. daily for periods up to 8 days had no action on liver repair after administration of CCl_4 or partial hepatectomy (Levvy *et al.* 1948). It failed to modify the increase in uterine weight and glucuronidase activity observed in ovariectomized mice during liver regeneration (Kerr *et. al.* 1949). In infant mice, intraperitoneal injection of 2 g. saccharic acid/kg. daily or the addition of 3% potassium hydrogen saccharate to the solid diet had no effect on normal growth after as long as 3 weeks.

Attempted synthesis of o-aminophenyl-glucuronide by β-glucuronidase

Florkin, Crismer, Duchateau & Houet (1942) claim to have demonstrated conjugation of borneol (saturated solution) with glucuronic acid (0·01 M) in presence of ox-spleen glucuronidase. At the end of the incubation period, free glucuronic acid was removed with copper sulphate and calcium hydroxide and glucuronic acid in combination was estimated by the Tollens colour reaction. Only a small fraction of the total glucuronic acid present was in the combined form, even after incubation for several days. The use of *o*-aminophenol as the aglycone in demonstrating glucuronide synthesis (Levvy & Storey, 1949) has the advantage that in the final reaction traces of the conjugate give a pink colour which is never seen in controls. No formation of aminophenylglucuronide was detected in experiments in which the free phenol was incubated with glucuronic acid in the presence of concentrated preparations of mouse liver glucuronidase.

D-Glucurone was present in final concentrations varying from 0·4 to 0·0125 M in 0·05 M-citrate buffer at pH 5·2, or 0·05 M-phosphate buffer at pH 7·4, containing 0·0025% *o*-aminophenol, 0·001 M-ascorbic acid, and the enzyme. The mixture was shaken for periods of 2 and 22 hr. at 37°. In the longer-term experiments, the incubation flasks were filled with N_2 to prevent oxidation of the free phenol.

When liver slices were replaced in the procedure of Levvy & Storey (1949) by crude liver homogenate, no glucuronide synthesis was detected.

DISCUSSION

Considerable difficulties were encountered in determining K_i, the dissociation constant for the inhibitor-enzyme complex, in the case of saccharic acid and β-glucuronidase, but it is considered that the value of 6×10^{-5} M finally arrived at is at least as reliable as values quoted for K_m, the dissociation constant of the substrate-enzyme complex, in the hydrolysis of biosynthetic glucuronides by glucuronidase. Figures available for K_m are as follows: phenylglucuronide, 0·0035 M (Kerr *et al.* 1948); bornylglucuronide, 0·01 M, methylglucuronide, 0·004 M, and oestriolglucuronide, 0·0005 M (Fishman, 1939); phenolphthaleinglucuronide, 0·00005 M (Talalay, Fishman & Huggins, 1946). Saccharic acid has a higher affinity for glucuronidase than all except one of these glucuronides. Changing the carboxyl at $C_{(6)}$ in saccharic acid to a primary alcohol group to give gluconic acid, or changing the configuration to give mucic acid, resulted in considerable diminution of the inhibitory power. The effect of glucuronic acid on the hydrolysis of phenylglucuronide by the enzyme may have been inhibition in the usual sense or a mass action effect. Hydrolysis of a glucuronide by glucuronidase is known to result in formation of free glucuronic acid (Levvy, 1948).

The failure of saccharic acid in large doses to modify liver regeneration after damage, or growth in infant mice may indicate that the enzyme is not directly concerned in cell division, but the results are capable of explanation in other ways. Saccharic acid may be too rapidly metabolized or excreted to produce any perceptible changes *in vivo*. Alternatively, normal cell division may involve hydrolysis of a naturally occurring glucuronide with a much greater affinity for the enzyme than that of saccharic acid. Experiments designed to exclude the possibility that saccharic acid does not penetrate the intact cell were unsatisfactory on technical grounds, but the results, for what they were worth, suggested that penetration did occur. Preliminary results obtained by Dr J. G. Campbell (private communication) suggest that saccharic acid considerably retards hydrolysis of the glucuronide of '1-*ortho*-hydroxyphenylazo-2-naphthol' by frozen mouse kidney sections in the histochemical test of Friedenwald & Becker (1948).

As a result of the work of Levvy *et al.* (1948), it is no longer necessary to postulate a synthetic role for β-glucuronidase in the body (Fishman, 1940) in order to explain the changes in the activity of the enzyme which can be produced in various organs. The view that β-glucuronidase is not involved in glucuronide synthesis (Levvy, 1948) receives support from the failure of saccharic acid to influence formation of *o*-aminophenylglucuronide by mouse-liver slices, and of β-glucuronidase preparations to effect condensation of glucuronic acid with *o*-aminophenol.

Certain sex hormones are known to be excreted as glucuronides. The effect of administering saccharic acid on the metabolism of these compounds might repay investigation.

SUMMARY

1. Hydrolysis of phenylglucuronide by β-glucuronidase was strongly inhibited by saccharic acid. Closely related compounds were much less effective. Inhibition by saccharic acid was competitive, and a value of 6×10^{-5} M was obtained for K_i, the dissociation constant of the enzyme-inhibitor complex.

2. Saccharic acid had no marked effect on synthesis of *o*-aminophenylglucuronide by mouse liver slices.

3. Administration of large doses of saccharic acid to mice did not influence liver regeneration after damage or growth in infant animals.

4. No conjugation of *o*-aminophenol with glucuronic acid was observed after incubation in the presence of β-glucuronidase.

The authors are indebted to the following for the supply of substances for test as possible inhibitors of glucuronidase: Prof. E. L. Hirst, F.R.S., Prof. G. A. R. Kon, F.R.S., Dr E. G. V. Percival and Dr J. Madinaveitia. Thanks are also due to Miss L. M. H. Kerr for assistance with certain of the animal experiments, to Dr J. G. Campbell for histological examination of organs and to Mr D. Love for technical assistance.

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The Glucuronide-synthesizing System in the Mouse and its Relationship to β-Glucuronidase

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e β-glucuronidase activity of mouse liver, kidney uterus has been shown to reflect the state of pro-ration of the tissue (Levvy, Kerr & Campbell, 48; Kerr, Campbell & Levvy, 1949*a*). It would pear that β-glucuronidase is distinct from the zyme system responsible for the production of ajugated glucuronides in the body. The work of pschitz & Bueding (1939) suggests that this ocess is more complex than a simple condensation the aglycone with free glucuronic acid, and that it kes place in liver and kidney only. β-Glucuroni-se, on the other hand, is present to a greater or less ent in practically all animal tissues that have so been examined (see, for example, Oshima, 1934). runairatnam & Levvy (1949) found that glu-ronide synthesis by adult mouse-liver slices was appreciably impaired by saccharic acid in concentrations which caused almost complete inhibition of β-glucuronidase.

The ability of various mouse tissues to synthesize glucuronides has been studied and compared with their glucuronidase activity under conditions leading to changes in the latter.

EXPERIMENTAL

Measurement of glucuronide synthesis. The conversion of *o*-aminophenol to its glucuronide in sulphate-free bicarbonate Ringer solution was followed by the method of Levvy & Storey (1949). Except in the case of lung, the tissue was sliced, and an amount corresponding to not less than 10 mg. dry weight was taken for each estimation. If necessary, slices from more than one animal were pooled. Whenever possible, the estimation was done in quadruplicate in order to reduce the variable error in the procedure (see Levvy & Storey, 1949). In the case of lung, the intact lobes were used. Boyland & McDonald (1948) have shown that this is permissible for measurements of metabolism in lung from young adult mice.

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Results are expressed in the tables as μg. *o*-aminophenol conjugated/g. dry weight of tissue in 1 hr. Both male and female mice were studied, but sex did not have any apparent effect on values for glucuronide synthesis, and it is therefore not usually shown in the tables.

Glucuronidase activity. The activity of this enzyme in tissue extracts was determined by the method of Kerr, Graham & Levvy (1948), and results are shown in terms of glucuronidase units (G.U.)/g. moist tissue, where 1 G.U. liberates 1 μg. phenol in 1 hr. from 0·015 M-phenylglucuronide at 37° and pH 5·2. In the case of tissues in which the kinetics of hydrolysis of phenylglucuronide by the enzyme have not yet been studied, it was assumed that optimal conditions for hydrolysis resemble those found for liver, spleen and kidney (Kerr *et al.* 1948; Kerr *et al.* 1949*a*).

RESULTS

Comparison of glucuronide synthesis and glucuronidase activity in various tissues. Table 1 shows the glucuronide-synthesizing power of liver, kidney, spleen and lung in young and adult mice, and of two transplantable mouse tumours. In agreement with Lipschitz & Bueding (1939), the synthetic system was found only in liver and kidney, the latter being much the less active of the two tissues. The ability of kidney slices to synthesize glucuronides was considerably less in young mice than in adults. This was also true for liver, and Fig. 1 shows the development of the synthetic system in the liver of the growing mouse.

Table 1. *The glucuronide-synthesizing power and β-glucuronidase activity of various mouse tissues*

(When the mean is based on values for individual animals the standard error of the mean for the group is also shown. Figures in brackets are numbers of animals used.)

Tissue	Age of animal	*o*-Aminophenol conjugated (μg./g. dry weight)	Glucuronidase activity/g. moist weight (G.U.)
Liver	Adult	570±43 (41)	273±13 (23)*
Kidney	Adult	150±14 (4)	363±24 (11)*
	9 days	30 (4)	—
	5 days	—	793 (4)*
Lung	Adult	Nil (2)	185±19 (3)
	9 days	Nil (3)	316±22 (3)
Spleen	Adult	Nil (3)	636±70 (23)*
	9 days	Nil (4)	—
	5 days	—	3245 (4)*
Sarcoma (Crocker 180)	Adult	Nil (2)	433±44 (6)
Carcinoma (Imperial Cancer Research Fund 2146)	Adult	Nil (2)	751±75 (6)

* Quoted from Levvy *et al.* 1948.

Also shown in Table 1 are figures for the glucuronidase activity of the various tissues, some quoted from earlier work from this Department, and others especially determined for present purposes. The distribution of this enzyme obviously bears no relation to that of the synthetic system. The high glucuronidase activity of organs from young mice compared with those from normal adults, already noted for liver, kidney, spleen and uterus (Levvy *et al.* 1948; Kerr *et al.* 1949*a*), is also seen in lung. The change in liver glucuronidase activity has now been fully studied from birth until maturity, and the results are shown in Fig. 2. This graph was compiled from results collected over a considerable period of time with four different strains of mice. The glucuronidase activity of the young mice at a given age was remarkably constant for the different strains and average figures for adults of different strains were indistinguishable from each other.

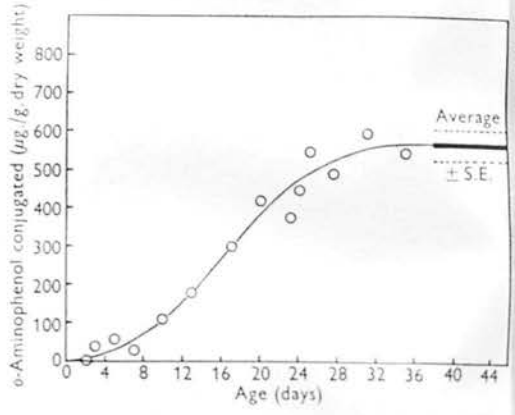

Fig. 1. The development of the glucuronide-synthesizing system, as measured by the conjugation of *o*-aminophenol, in the liver of the growing mouse (own mixed colony).

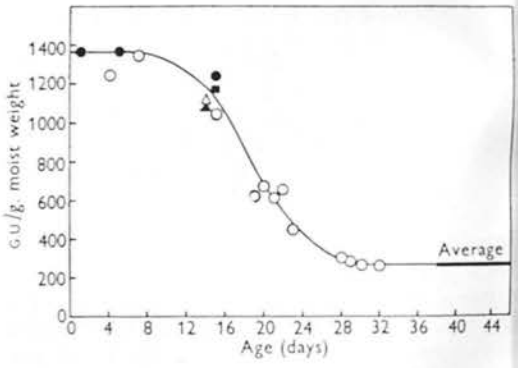

Fig. 2. The change in glucuronidase activity in the liver of the growing mouse: ■, strain C57; △, strain A; ▲, strain CBA; ●, own mixed colony, August 1947; ○, own mixed colony, February 1949.

n agreement with the observations of Fishman & yan (1947) on human tissues, both mouse tumours e high in glucuronidase activity when compared h most normal adult tissues.

he role of glucuronidase in glucuronide synthesis. possibility had to be considered that the activity he glucuronide-synthesizing system in the liver s not vary with age, but that the hydrolytic vity of β-glucuronidase at any given age deter- es the net amount of synthesis which can be sured (compare Figs. 1 and 2). It has already n shown (Karunairatnam & Levvy, 1949) that charate in a concentration of 10^{-2}M causes ost complete inhibition of glucuronidase in liver idney extracts, but has no appreciable effect on curonide synthesis by adult mouse-liver slices. many of the experiments summarized in Table 1, litional determinations of glucuronide synthesis e made in presence of 10^{-2}M-saccharate. This stance caused no increase in the synthetic power nfant liver or adult kidney, nor did its presence l to glucuronide synthesis by lung, spleen or iour slices. Failure of saccharate to penetrate the would appear to be excluded, since Campbell & y (1949) have found that it inhibits hydrolysis lucuronides by mouse kidney in the two histo- mical tests of Friedenwald & Becker (1948). It ns, therefore, that the glucuronidase activity of ssue has no bearing on its ability to synthesize curonides.

he effect of various measures on the glucuronide- thesizing system in vivo. Various measures leading hanges in the state of proliferation of mouse liver e examined for their action on the glucuronide- thesizing system. The results are shown in Table 2, it can be seen that in no instance was there a ctable change in the activity of the synthetic em. Under similar conditions, a rise in glucu- dase activity to two or three times the normal e was seen after partial hepatectomy, or in- ion of menthol, carbon tetrachloride or oestrone vvy *et al.* 1948; Kerr *et al.* 1949*a*). Colchicine in dose shown causes no change in the normal curonidase activity of liver, but it does prevent rise in activity which follows such measures as tial hepatectomy. In the smaller dose, sorbic l behaves like colchicine, but the larger dose mg./kg.) causes a profound depression in liver kidney glucuronidase activity in normal mice. effects of colchicine and sorbic acid on glucu- dase activity appear to be related to their ons as inhibitors of mitosis (Kerr, Campbell & vvy, 1949*b*).

he effect of various compounds on the synthesis of minophenylglucuronide in vitro. Four of the pounds examined for their action on the glucu- de-synthesizing system *in vivo* were tested *in* for their effect on the conversion of *o*-amino- phenol (0·00023 M) to its glucuronide by surviving liver slices from normal mice (Table 3). The fifth compound listed in Table 2, oestrone, was too sparingly soluble in water to permit its study in the present experiments.

Table 2. *The effect of various measures on the glucuronide-synthesizing system in mouse liver*

(When the mean is based on values for individual animals the standard error of the mean for the group is also shown. Figures in brackets are numbers of animals used.)

Treatment	Dose (g./kg.)	Days after treatment	*o*-Aminophenol conjugated/g. dry weight (μg.)
None	—	—	570±43 (41)
(−)-Menthol, intraperitoneally in olive oil	0·33	1	560±40 (3)
		3	640 (2)
		6	650 (2)
Carbon tetrachloride, subcutaneously in olive oil	5·33	1	560±170 (3)
		3	640±200 (3)
		7	640±150 (3)
Partial hepatectomy	—	3	670±60 (6)
		7	650±50 (6)
		10	610±80 (6)
Oestrone, subcutaneously in olive oil (ovariectomized mice)	0·0017	4	580±70 (3)
Colchicine, subcutaneously in aqueous solution	0·0015	1	450±80 (6)
Sorbic acid, subcutaneously in aqueous solution	0·24	4	440±70 (12)
	0·16	4	540±120 (6)

Table 3. *The effect of various compounds on the synthesis of o-aminophenylglucuronide by mouse-liver slices*

Compound	Concentration (M)	*o*-Aminophenol conjugated (μg./g. dry weight) In controls	In presence of compound	Inhibition (%)
Sorbic acid	0·01	380	50	87
	0·01	970	370	62
	0·005	970	500	49
Colchicine	0·01	380	140	63
	0·01	530	190	64
	0·005	530	270	49
Carbon tetrachloride	0·0015	490	520	−6
	0·0015	660	520	21
(−)-Menthol	0·001	450	120	73

Colchicine, sorbic acid and menthol were added as aqueous solutions (if necessary, after pH adjustment) during the preparation of the bicarbonate Ringer solution. In the case of CCl_4, the Ringer solution was made saturated with the compound. In all experiments, controls were done with untreated slices from the same animal. Each determination was done in quadruplicate, and the standard error of the mean is thus about 10% (Levvy & Storey, 1949).

As can be seen from Table 3, in the presence of colchicine, sorbic acid or menthol there was a drop in the conversion of *o*-aminophenol to its glucuronide which was outside the range of error. Inhibition was almost 50% with 0·005M-colchicine or sorbic acid, and 73% with 0·001M-menthol. Carbon tetrachloride had no appreciable effect in the highest concentration possible (0·0015M). None of the four compounds in question interfered in the colour reaction for *o*-aminophenylglucuronide.

DISCUSSION

From the results of the experiments described above, it seems clear that there are at least two distinct enzyme systems in the mouse concerned with the metabolism of the conjugated glucuronides. One of these, β-glucuronidase, is present in practically every tissue, and its action is probably entirely hydrolytic. The activity of this enzyme in a tissue varies with the degree of cell division in progress. The other enzyme system is responsible for the synthesis of glucuronides, and is probably complex. It has so far been found only in liver and, to a smaller extent, kidney. The activity of this enzyme system in liver is not altered by measures causing changes in the state of proliferation of the tissue. The ability of liver or kidney to synthesize glucuronides does, however, vary with the age of the animal. At birth, the activity of the synthetic system is small or nil, and it only reaches its ultimate value when the animals are 4 or 5 weeks old. Taken together, the two enzyme systems may provide a mechanism for regulating the transport, action and excretion of physiologically active, glucuronidogenic compounds, such as oestriol. Alternatively, their function may be to provide free glucuronic acid or a transformation product for building up into more complex mol cules.

Since menthol is known to form a glucuronide the presence of surviving liver slices (Lipschitz Bueding, 1939), its depressant action on the sy thesis of *o*-aminophenylglucuronide *in vitro* is pro ably the result of competition with *o*-aminophen rather than genuine inhibition of the synthet mechanism. The inhibitory actions of sorbic acid ar colchicine on glucuronide synthesis *in vitro* a difficult to interpret at present. The overall sy thetic process is known to be adversely affected b other agents, such as cyanide, fluoride and iod acetate (Lipschitz & Bueding, 1939), azide and su phate (Dr I. D. E. Storey, private communicatio but their mode of action is in most cases still obscu

SUMMARY

1. The glucuronide-synthesizing system in t mouse was found only in liver and, to a small extent, kidney.

2. Measures causing changes in the state of pr liferation of liver had no effect on the activity of t glucuronide-synthesizing system.

3. The activity of the synthetic system was almo nil at birth, and only reached its ultimate value wh the mice were more than 1 month old.

4. The effects of various compounds on gluc ronide synthesis *in vitro* were studied.

5. On the basis of this work, the enzyme syste responsible for glucuronide synthesis can be clear distinguished from β-glucuronidase.

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Glucuronidase and Glucuronide Synthesis. By M. C. Karunairatnam and G. A. Levvy. (*Department of Biochemistry, University of Edinburgh*)

In view of the relationship observed between the activity of β-glucuronidase in a tissue and the state of proliferation (Levvy, Kerr & Campbell, 1948) it was considered important to find an inhibitor for this enzyme. Of many compounds studied, including several 'growth inhibitors', by far the most effective was saccharic acid. Almost complete inhibition of the hydrolysis of phenol glucuronide (0·015 M) by mouse liver or kidney preparations was obtained with 10×10^{-3} M-saccharate, and 50% inhibition with 2×10^{-4} M. Closely related compounds were much less effective than saccharic acid. No evidence has so far been obtained of any action, even in massive doses, of saccharic acid on growth processes in the mouse.

It has been suggested, without any direct evidence, that β-glucuronidase is responsible for glucuronide formation in the body (Fishman, 1940). Levvy & Storey (1948) have developed a method for measuring glucuronide synthesis by mouse-liver slices. In concentrations up to 10^{-2} M, saccharic acid had no appreciable effect on this process.

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Glucuronide Synthesis and Cell Proliferation. By M. C. Karunairatnam, Lynda M. H. Kerr and G. A. Levvy. (*Department of Biochemistry, University of Edinburgh*)

In view of the relationship found to exist between the state of proliferation and the β-glucuronidase activity of mouse liver, kidney and uterus (Levvy, Kerr & Campbell, 1948; Kerr, Campbell & Levvy, 1949), the possibility that the ability of mouse liver and other tissues to synthesize glucuronides may also vary with the degree of cell division in progress was investigated by the method of Levvy & Storey (1949).

Unlike β-glucuronidase activity, the ability to synthesize glucuronides seemed to be confined largely to liver. Kidney, the only other tissue found to carry out the synthesis, was much less active than liver. This is in agreement with the results of Lipschitz & Bueding (1939).

During the period of enhanced glucuronidase activity in liver which follows partial hepatectomy or administration of carbon tetrachloride or menthol, the ability to synthesize glucuronides remained normal. It was also unchanged during the profound depression of glucuronidase activity which follows sorbic acid administration. Tumours with high glucuronidase activity were devoid of synthetic power, as were livers from mice 2–3 days old. It seems clear that, unlike β-glucuronidase, the enzyme system causing glucuronide synthesis does not vary in activity with the state of proliferation of a tissue, and that the two enzyme systems are quite distinct (see Levvy, 1948; Karunairatnam & Levvy, 1949). The synthetic power of mouse liver remained very small for the first week after birth. It then rose slowly till at the fourth week the activity had reached the average value for normal adults. The β-glucuronidase activity of liver in the first week after birth was six times the value found in adults. It then fell steadily to become constant when the mice were 4 weeks old.

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